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TITLE: Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction

PRINCIPAL INVESTIGATOR: Nadine Hempel, PhD

CONTRACTING ORGANIZATION: The Pennsylvania State University
Hershey, PA 17033

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6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our work addresses the hypothesis that mitochondrial dysfunction plays a role in the etiology and chemoresistance of epithelial ovarian cancers. We are focusing on the role of the fission protein Drp1 in this context. Specifically we discovered that expression of a low molecular weight Drp1 variant is associated with mitochondrial fission/fusion defects. Mass spec and RNA sequencing analysis has revealed that the low molecular weight (LMW) isoform of Drp1 does not arise as a consequence of alternate transcriptional promoter use, but may be dependent on an alternate variable domain and C-terminal truncation. We are interrogating the role of short Drp1 as a dominant negative fission protein and are investigating its binding affinity to mitochondria and interaction with fission accessory proteins. Investigations on the function of this protein in mediating mitochondrial dysfunction and chemoresistance are ongoing. We have identified that expression of LMW Drp1 is detected in the majority of high grade serous ovarian cancer cells isolated from patient ascites, and that this is associated with hyperfused mitochondria, indicating that this is a clinically relevant observation that could affect a majority of ovarian cancer cases.

15. SUBJECT TERMS
Drp-1, DNM1L, mitochondria, mitochondrial fission, ovarian cancer

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1. INTRODUCTION:

Epithelial Ovarian Cancer (EOC) remains the most deadly gynecological malignancy, characterized by high rates of relapse and chemoresistance. Metabolic screening of EOC cell lines and patient ascites-derived tumor cells revealed that a distinct subgroup of EOC demonstrate severe mitochondrial dysfunction, which is accompanied by hyperfused mitochondria and expression of a low molecular weight variant of the mitochondrial fission protein Drp1. Our data suggest that compromised mitochondrial function and fission/fusion dynamics may be a hallmark of a previously unidentified subgroup of highly chemoresistant EOCs and that this is associated with aberrant expression of the fission protein Drp1. This work addresses the hypothesis that Drp1-dependent mitochondrial dysfunction plays a role in the etiology and chemoresistance of a distinct subgroup of epithelial ovarian cancers. Our major aims of this work are to 1. establish the clinical significance of mitochondrial dysfunction in a cohort of ovarian cancer patients, 2. elucidate the mechanistic consequences of Drp1 splice variant expression on EOC mitochondrial function, metabolism and chemoresistance, and 3. investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs. These studies have implications for the future development of Drp1 variants as biomarkers in the screening and identification of a subgroup of highly chemoresistant EOCs. The reliance of these cells on alternate metabolic pathways as a consequence of their mitochondrial deficiency makes this subgroup of EOCs a target for anti-metabolism based therapies.

2. KEYWORDS:

Drp-1, DNM1L, mitochondria, mitochondrial fission, ovarian cancer

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project are listed below in the format of the original SOW:

Specific Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC

- **Major Task 1** Test if mitochondrial dysfunction is a common clinical phenotype of highly chemoresistant EOCs:
    - *Milestone #1: IRB and HRPO approval received – COMPLETED July 2016*
        - Subtask 1: Obtain IRB approval & submit necessary documents for HRPO review. IRB (STUDY4648; 03-29-2016) and HRPO (A-19540; 29-07-2016) approval received
    - *Milestone #2: Completion of statistical analysis of patient mitochondrial dysfunction and association with clinical parameters – to be completed by May 2018*
        - Subtask 2: Isolation and culturing of cells from patient ascites. ONGOING
        - Subtask 3: Statistical Analysis patient data: - to be completed end of year 2, following collection of all patient samples
- **Major Task 2** Determine the identity and frequency of expression of Drp1 splice variants, and examine their association with mitochondrial dysfunction and chemoresistance.
    - *Milestone #3: Major Drp1 splice variant in mitochondrial deficient EOCs identified – COMPLETED - December 2016*
        - Subtask 1: Identify Drp1 splice variants by mass spec.
        - Subtask 2: Identify Drp1 splice variants by RNA Seq.

        - Subtask 3: Design primers for identification of Drp1 splice variants in patient specimens.
    - *Milestone #4: Completion of statistical analysis of patient mitochondrial dysfunction and association with Drp1 expression – to be completed by May 2018*
        - Subtask 4: Assess Expression of Drp1 variants in patient derived EOC RNA samples. ONGOING
        - Subtask 5: Assess mitochondrial morphology and chemoresitance of patient derived EOC RNA samples. ONGOING.
        - Subtask 6: Statistical Analysis of patient data, association of Drp1 expression with mitochondrial function, chemoresistance profiles and histological subtype . TO BE COMPLETED IN YEAR 2, following collection of all patient samples
- **Major Task 3** Test if DNM1L copy number alterations correlated with Drp1 expression
    - *Milestone #5: Completion of all data analysis of clinical specimen – to be completed by May 2018*
        - Subtask 1: Assess DNM1L copy number in patient derived EOC RNA samples. ONGOING
    - *Milestone #6: Manuscript submission of data on mitochondrial function and & association with Drp1 expression & DNM1L copy number alterations – to be completed by May 2018*

Specific Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on EOC mitochondrial function, metabolism and chemoresistance.

- **Major Task 4** Test if identified Drp1 transcript variants act as dominant negative fission proteins.
    - *Milestone #7: identification of Drp1 variant function on mitochondrial fission and function – 80% achieved, to be completed August 2017*
        - Subtask 1: Cloning of Splice variants. ONGOING.
        - Subtask 2: Experiments of recombinant Drp1 constructs on mitochondrial fission using confocal microscopy & TEM & Seahorse extracellular flux analysis. ONGOING.
    - *Milestone #8: identification of Drp1 splice variant protein-protein interaction - 60% achieved, to be completed September 2017*
        - Subtask 3: protein-protein interaction studies. ONGOING
- **Major Task 5** Determine if Drp1-mediated mitochondrial dysfunction aids in chemoresistance by disrupting programmed cell death.
    - *Milestone #9: Identification of Drp1 variant expression on programmed cell death and chemoresistance and finalization of data analysis – 30% achieved, to be completed December 2017*
        - Subtask 1: Effects of recombinant Drp-1 constructs on apoptosis & autophagy markers in response to chemotherapeutics. ONGOING
        - Subtask 2: Effects of recombinant Drp-1 constructs on Bax interaction. - to commence June 2017
    - *Milestone #10: Manuscript submission of data Drp1 variants as potential dominant & negatives in the regulation of mitochondrial fission and programmed cell death – to be completed January 2018*

- **Major Task 6** Assess if Drp1-mediated mitochondrial dysfunction contributes to chemoresistance by eliciting cellular DNA damage response.
    - *Milestone #11: Identification of Drp1 variant expression on DNA damage response and chemoresistance & finalization of data analysis – to commence June 2017 and to be completed February 2017*
        - Subtask 1: Effects on ECCR1 protein stability in response to recombinant Drp1 variant expression & assessment of Drp1 expression and other DDR protein levels. TO BE COMPLETED IN YEAR 2
        - Subtask 2: effects of recombinant Drp1 splice variants on DDR signaling. TO BE COMPLETED IN YEAR 2
        - Subtask 3: effects of ATM/ATR inhibitors on Drp1 variant expressing cell lines and EOC specimen. TO BE COMPLETED IN YEAR 2
    - *Milestone #12: Manuscript submission of data Drp1 variants as potential dominant negatives in the regulation of mitochondrial fission and programmed cell death – to commence February 2018 and be completed by March 2018*

Specific Aim 3: To investigate alternate therapeutic strategies for mitochondria-deficient EOCS

- **Major Task 7** Determine the alternate metabolic pathways used by mitochondria defective cells.
    - *Milestone #13: correlation of metabolic inhibitor response with EOC metabolic profiles and mitochondrial dysfunction - 20% achieved, to be completed May 2018*
        - Subtask 1: Growth studies of EOCs under nutrient limitations. ONGOING
        - Subtask 2: Glycolytic flux/Bioenergetics measurements of EOC specimens. ONGOING
- **Major Task 8** Assess if disruption of alternate metabolic pathways by metabolism-based inhibitors represents a novel therapeutic strategy to target mitochondria-defective cells.
        - Subtask 1: Dose response curves to metabolic inhibitors Ovca cell lines TO BE COMPLETED IN YEAR 2
        - Subtask 2: Metabolic inhibitor studies on cell viability of EOC specimens TO BE COMPLETED IN YEAR 2
    - *Milestone #12: Manuscript submission describing the relevance of metabolic targeting of mitochondria defective EOCs – – to commence March 2018 and be completed by May 2018*
    - *Milestone #13: Submit grant application to NCI R01 for follow up work – 50% achieved, R01 to be submitted for June or October deadline*

What was accomplished under these goals?

Below are the major results obtained during the reporting period:

Specific Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC

Objective: Determine the identity and frequency of expression of Drp1 splice variants, and examine their association with mitochondrial dysfunction and chemoresistance (Task 2)

Activities & Outcomes:

- We continue to collect patient ascites samples and have identified that expression of LMW Drp1 is primarily expressed in cells of high grade serous adenocarcinoma origin (HGSA, Figures 1A & B), with 50% of HGSA samples displaying expression of LMW-Drp1 that exceeds expression of full length Drp1 (Figure 1B). Strong expression of LMW-Drp1 correlates with hyperfusion and aggregation of mitochondria (Figure 1C).

- Our major effort during the past year focused on investigating the molecular mechanism resulting in LMW Drp1 expression.
- RNA seq, RT-PCR and 5'RACE analysis was performed to determine if this variant arises as a consequence of alternate 5'promoter use or alternate splicing (Figure 1D). Using 5'RACE we determined that alternate promoter use likely does not contribute to the generation of a shorter transcript, which was confirmed by RNA seq data. Lack of alternate start sites were further confirmed by Mass Spec analysis.
- Instead of an alternate start site we discovered differential splicing of exon 16 and 17 of the "variable" region of Drp1. RNA seq and RT-PCR with primers targeting differentially spliced exons 16 and 17 demonstrated that OVCA420 cells, which predominantly express LMW Drp1 have a lower proportion of transcripts including exon 16, compared to OVCA433 cells which predominantly express full length FL Drp1 (Figure 1D). This was also confirmed in patient derived epithelial ovarian cancer (EOC) cells samples obtained from ascites, where specimens displaying high expression of LMW-Drp1 had a lower ratio of Exon 16 transcript expression (Figure 1D). This region is of interest has it has been implicated with intermolecular Drp1 binding and binding of Drp1 to mitochondria via mitochondrial receptors such as Drp1 (Figure 1F). This is under current investigation using mutation construct of Drp1 with variable exon 16/17 expression (Aim 2).
- Using Mass spec analysis we confirmed that the low molecular weight (LMW) 60kDa variant is Drp1 and may have a truncation in the C-terminal domain (Figure 1E & F).

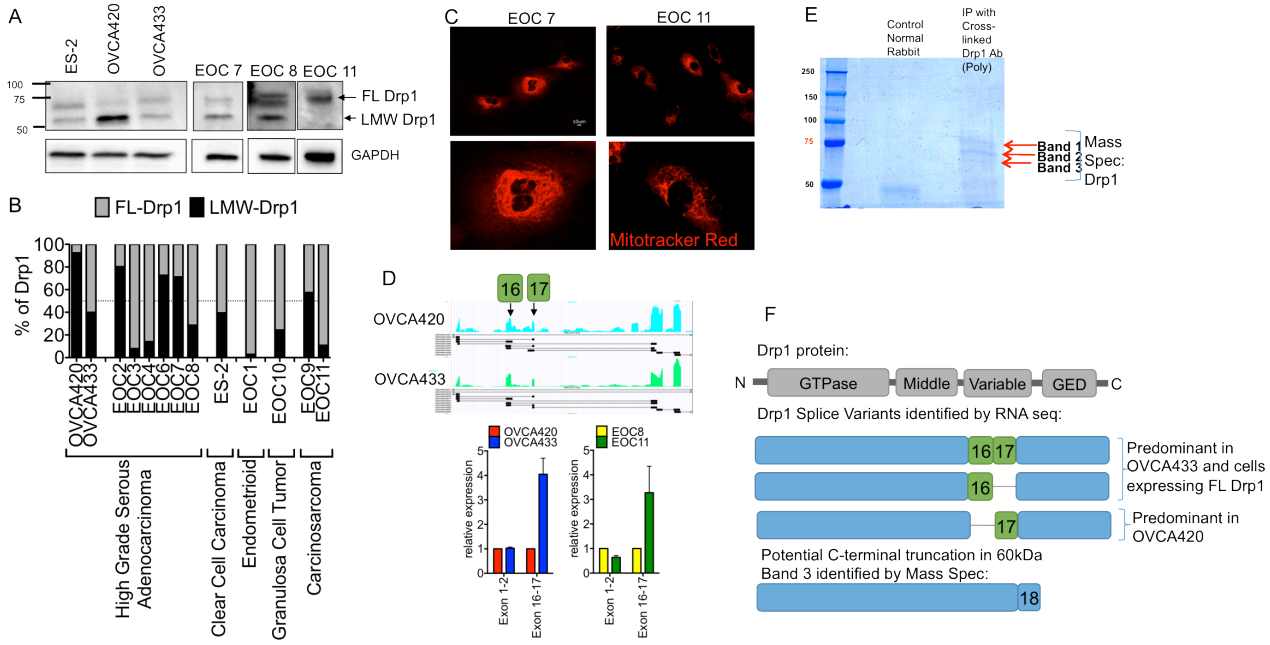

Figure 1: **A.** LMW Drp1 protein expression in cell lines (OVCA420, OVCA433 & ES-2) and patient ascites derived epithelial ovarian cancer cells (EOC, western blotting). **B.** Ratio of full length (FL) to Low Molecular Weight (LMW)-Drp1 expression in cell lines and EOC samples, grouped by histological classification. **C.** EOC7 cells expressing LMW-Drp1 demonstrate hyperfused mitochondria compared to EOC11 cells, which predominantly express FL-Drp1 (Mitotracker CMX-ROS staining). **D.** LMW-Drp1 expression correlates with decreased exon 16 presence in mRNA transcripts (assessed by RNA seq and RT-PCR) **E.** Mass Spec analysis of Drp1 variants was carried out following IP with Drp-1 polyclonal antibodies (Wistar Mas Spec core) and confirmed the presence of Drp1 peptide sequences in all three bands. Band 3 is LMW Drp1. A lack of

coverage in the C-terminal domain suggests C-terminal truncation in the LMW variant. **F.** Alternately spliced Exons 16 and 17 are located in the variable domain responsible for Drp1 intermolecular interaction.

- Moreover, we discovered that LMW-Drp1 was primarily expressed in cells grown under attached proliferating conditions, and that full length Drp1 expression was restored when cells were grown in anchorage independent conditions (Figure 2A). This was accompanied by increased fission in anchorage independent spheroid conditions, as demonstrated by shorter mitochondrial fragments in TEM and with increased mito/autophagy, as demonstrated by LC3B western and autophagosomes visible by TEM (Figure 2B&C)

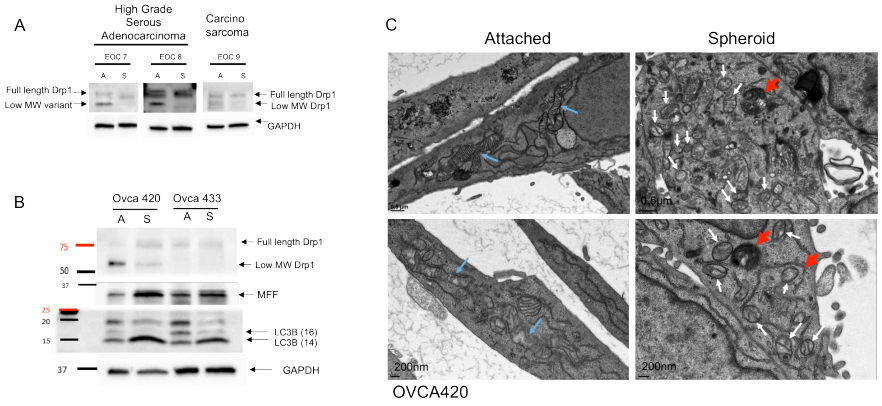

Figure 2: Strong expression of LMW Drp1 is observed in patient ascites derived tumor cells from HGSA histological subtypes (**A**). LMW Drp1 expression is decreased and FL Drp1 expression increased when cells are grown in anchorage independent spheroids (S) in Ultra low attachment plates for 3 days, compared to attached conditions (A). Increased FL Drp1 expression is associated with autophagy markers LC3B (**B**) and shorter mitochondria as visualized by TEM (**C**). Blue arrows indicate elongated and hyperfused mitochondria in OVCA420 cells in attached conditions, while arrows indicate smaller mitochondria in spheroid conditions. Red arrows indicate auto phagosomes.

Specific Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on EOC mitochondrial function, metabolism and chemoresistance.

Objectives : Test if identified Drp1 transcript variants act as dominant negative fission proteins (Major Task 4). Determine if Drp1-mediated mitochondrial dysfunction aids in chemoresistance by disrupting programmed cell death (Major Task 5). Assess if Drp1-mediated mitochondrial dysfunction contributes to chemoresistance by eliciting cellular DNA damage response (Major Task 6).

Activities & Outcomes:

- Using mitotracker and immunofluorescence imaging we have shown that Drp1 mislocalizes to the cytoplasm in OVCA420 cells, suggesting that LMW Drp1 influences Drp1 binding to mitochondria (Figure 3).
- Current work focuses on optimizing co-IP studies for interaction of mitochondrial receptors, such as Mff with Drp1.
- Following identification of low molecular weight Drp1 by mass spec we are now in the process of cloning deletion mutants with differential expression of exons 16 and 17 and a truncation of the C-terminus (Figure 1D). These will be expressed in OVCA420 CRISPR Drp1 knock down cells. Knock down of Drp1 in OVCA420 and OVCA433

cells following CRISPR/Cas9 transfections and selection is currently being verified by sequencing.

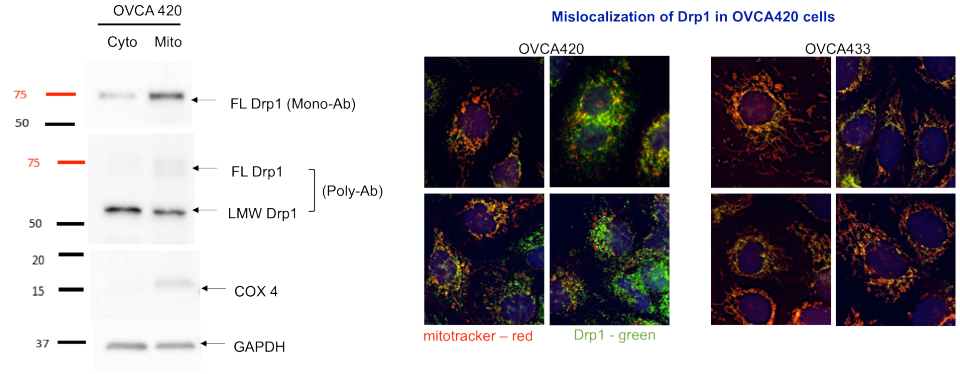

Figure 3: LMW Drp1 is found in the cytoplasm and fails to associate with mitochondria at distinct fission puncta in OVCA420 cells when compared to OVCA433 cells which express predominantly FL Drp1.

Other goals achieved: Based on data gathered under this funding mechanism we are hoping to extend this work to investigate the transient changes in Drp1 expression in anchorage independent conditions and how this relates to fusion and fission following matrix detachment. We are currently preparing an R01 application for submission to the NIH.
Stated goals not met: We have been generally on track with experiments based on our Milestones of the SOW. However, delays were experienced in getting RNASeq and Mass spec data, due in part to trouble shooting of purification and preparation of proteins for mas spec analysis and turn-around time in obtaining data for RNA seq and mass spec from core facilities. This has somewhat delayed our anticipated verification of the Drp1 variants (Milestone #3). In addition, there was a 4 month delay in getting IRB and HRPO approval, which somewhat delayed collection of patient ascites samples after the study was first funded. Collection of patient samples will continue until the end of the study period to meet the target enrollment goal.

What opportunities for training and professional development has the project provided?

- The PI, Dr. Nadine Hempel, and postdoc, Dr. Dong Hui Shin, attended the *NIH special conference on mitochondria in May 2016* in Bethesda MD. At this meeting both were exposed to the most recent developments and cutting edge research techniques used in mitochondrial research.
- Dr. Hempel attended the *Marsha Rivkin symposium on Ovarian cancer* in September 2016, Seattle WA to gain exposure to the latest developments in ovarian cancer research.
- Dr. Dong-Hui Shin has acquired a wide new skill set in mitochondrial biology and ovarian cancer through technical training during this award period. For example he has been trained on the use of transition electron microscopy (TEM) and protein purification techniques for preparation and analysis of Drp1 variants by mass spec. He has been regularly meeting with Dr. Hempel, who has mentored Dr. Shin throughout the grant period. Dr Shin has also benefitted from mentorship of Dr. H.G. Wang, a collaborator on the project, and has taken advantage of career development seminars through the College of Medicine, Penn State Hershey

How were the results disseminated to communities of interest?

- Posters were presented at the following meetings to disseminate our recent findings from this project:
    - *NIH/NHLBI special conference on mitochondria, May 2016*, Bethesda MD
    - *Marsha Rivkin & AACR symposium on Ovarian cancer,* September 2016, Seattle WA

What do you plan to do during the next reporting period to accomplish the goals?
We will address the remaining goals of our proposal as outlined in the SOW.
Specific Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC

- Test if mitochondrial dysfunction is a common clinical phenotype of highly chemoresistant EOC (Major Task 1).
    - As we are continuing to receive EOC samples form ascites, we are examining their Drp1 expression and correlating this to mitochondrial morphology and bioenergetics phenotypes using Seahorse extracellular flux analyzers. Moreover we are examining the correlation to chemoresistance
    - Final data analysis of this will be completed at the end of year 2.
- Test if DNM1L copy number alterations correlated with Drp1 expression (Major Task 3)
    - We are continuing collecting DNA samples of patents and will undertake copy number analysis and statistical association studies at the end of 2 year of the study.

Specific Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on EOC mitochondrial function, metabolism and chemoresistance.

- Test if identified Drp1 transcript variants act as dominant negative fission proteins. (Major Task 4)
    - We are in the process of cloning various Drp1 constructs into dTomato expression and eGFP expression vectors and are *making a* Drp-1 CRSPR OVCA420 and OVCA433 knockdown cell lines for re-expression of full length and Low molecular weight variants for continued studies of Aim 2. Sequencing is under way to confirm successful knock out.
    - Using these recombinant Drp-1 constructs and cell lines, we will assess if short Drp1 acts as a dominant negative on fission, by TEM and mitotracker analysis
- Determine if Drp1-mediated mitochondrial dysfunction aids in chemoresistance by disrupting programmed cell death (Major Task 5)
    - The above constructs and cell lines will be used to determine the role of LMW Drp1 in inhibiting apoptosis & autophagy markers in response to chemotherapeutics cisplatin and Taxol, as well as authphagy inhibitors.
    - Mechanistically we will examine if recombinant Drp-1 constructs affect binding to apoptosis protein Bax interaction.
- Assess if Drp1-mediated mitochondrial dysfunction contributes to chemoresistance by eliciting cellular DNA damage response. (Major Task 6)
    - The Effects on ECCR1 protein stability and other DDR protein levels will be examined in response to recombinant Drp1 variant expression.
    - The effects of recombinant Drp1 mutants will be monitored on DDR signaling, focusing on ATM and ATR.
    - The effects of ATM/ATR inhibitors on Drp1 variant expressing cell lines and EOC specimen will be tested

Specific Aim 3: To investigate alternate therapeutic strategies for mitochondria-deficient EOCS

- Determine the alternate metabolic pathways used by mitochondria defective cells. (Major Task 7)
    - We will correlate if metabolic inhibitor response is associated with EOC metabolic profiles and mitochondrial dysfunction as determined by Seahorse Bioenergetic flux analysis.
    - These will be accompanied by Growth studies of EOCs under nutrient limitations.
- Assess if disruption of alternate metabolic pathways by metabolism-based inhibitors represents a novel therapeutic strategy to target mitochondria-defective cells. Major Task 8
    - Dose response curves will be carried out with to metabolic inhibitors on Ovca cell lines and EOC specimens.

Our major milestones to be achieved in year 2 are:

- Identification of Drp1 variant function on mitochondrial fission and function
- Identification of Drp1 splice variant protein-protein interaction
- Identification of Drp1 variant expression on programmed cell death and chemoresistance and finalization of data analysis
- Identification of Drp1 variant expression on DNA damage response and chemoresistance
- Completion of all data analysis of clinical specimen, including statistical analysis of patient mitochondrial dysfunction and association with clinical parameters
- correlation of metabolic inhibitor response with EOC metabolic profiles and mitochondrial dyfunction
- Manuscript submission of:
    - Data on mitochondrial function and association with Drp1 expression & DNM1L copy number alterations.
    - Manuscript submission of data Drp1 variants as potential dominant negatives in the regulation of mitochondrial fission and programmed cell death
    - Manuscript submission describing the relevance of metabolic targeting of mitochondria defective EOCs

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have made strides in our verification to demonstrate that expression of alternate variants of Drp1 are clinically relevant. In the coming year we will verify that these changes in Drp1 have functional consequences on ovarian cancer etiology. In addition, new data emanating from this work has opened new research avenues for the investigation of Drp1 regulation during different stages of ovarian cancer metastasis.

What was the impact on other disciplines?

The role of mitochondrial dynamics has far reaching consequences beyond the field of ovarian cancer, and the basic mechanisms of mitochondrial dynamics unraveled in our work have the potential to impact pathologies where mitochondrial dysfunction is a known driver of disease, including neurodegenerative diseases.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report (N/A)

Significant changes in use of biohazards and/or select agents

Nothing to Report (N/A)

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

- Hempel N, Trebak M. Crosstalk between calcium and reactive oxygen species signaling in cancer. *Cell calcium*. (2017) Jan 18 (in press), DoD funding acknowledged: yes

Books or other non-periodical, one-time publications.

Nothing to Report

Other publications, conference papers, and presentations.

- Shin DH, Dier U, Timmins PF, Kesterson J, Phaeton R, Hempel N Mitochondrial dynamics and dysfunction in ovarian cancer. September 2016, Rivkin Center for Ovarian Cancer and AACR – Ovarian Cancer Research Symposium, Seattle WA. Poster Presentation.
- Shin DH, Kim Yeon Soo, Dier Usawadee, Timmins PF, Yoon Yisang, Kesterson Joshua, Phaeton Rebecca and Hempel N. The role of mitochondria fission protein Drp1 in metastatic ovarian cancer. May 2016 The NHLBI/NIDDK Mitochondrial Biology Symposium, National Institutes of Health, Bethesda, Maryland. Poster Presentation.

- **Website(s) or other Internet site(s)**

  Nothing to Report

- **Technologies or techniques**

  Nothing to Report

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Nadine Hempel
Project Role:	P.I.
Researcher Identifier:	orcid.org/0000-0002-5574-8783
Nearest person month worked:	1
Contribution to Project:	**From Budget Justification:** 5% salary is requested for the

	PI. Dr. Hempel will oversee the intellectual aspect and conceptual designs of the project, including experimental design, data analysis, manuscript preparation, meeting presentations and providing guidance to the research team.
Name:	Dong Hui Shin
Project Role:	Postdoctoral Scholar
Researcher Identifier:	
Nearest person month worked:	11
Contribution to Project:	**From Budget Justification: TBD Postdoctoral Scholar** (12 calendar months) 100% salary is requested for a Postdoctoral Scholar to carry out the majority of research experiments proposed, including studies pertaining to the identification of Drp1 splice forms, metabolic profiling, cell culture assays, handling of ascites derived tumor cells and mitochondrial functional studies.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Drs. Hempel, Kesterson, Phaeton, Wang, and Warrick all had changes in their active other support. Please see below.

OTHER SUPPORT - Hempel, Nadine

ACTIVE PROJECTS - Previously Reported:
None

PROJECTS FUNDED SINCE LAST REPORT:
W81XWH-16-1-0117 (Hempel, Nadine) 5/1/2016-4/30/2018 0.60 cal mths
U. S. Army Medical Research and Development Command $250,000 DC/Yr
Contracting/Grants Officer: Joshua Disbennett, Grant Specialist, Grants Administration Office, 301-619-7349, Joshua.L.Disbennett.civ@mail.mil

Title:	Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction
Goals:	The goal of our study is to identify the clinical relevance and mechanism leading to mitochondrial dysfunction in ovarian cancer, and identify how this influences chemoresistance and ovarian cancer etiology.
Specific Aims:	Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC. Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on ovarian cancer mitochondrial function, metabolism and chemoresistance. Aim 3: To investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs.
Role:	P.I.

Overlap: *This is the project for which the progress report is being submitted.*

Pilot Study (Hempel, Nadine) 4/1/2017-3/31/2018 0.60 cal mths
Marsha Rivkin Center for Ovarian Cancer Research $75,000 DC/Yr
Contracting/Grants Officer: Kiran Dhillon, PhD, Director of Scientific Programs, Rivkin Center for Ovarian Cancer, (206) 215-2964, Kiran.Dhillon@swedish.org.
Title: Pre-clinical Investigation of High Dose Ascorbate IP Therapy
Goals: Using a combination of patient-derived samples, cell culture models, and in vivo studies, we will test the feasibility of high dose ascorbate IP therapy and interrogate the molecular mechanisms of action as it pertains to ovarian cancer cells in the IP cavity tumor environment.
Specific Aims: Aim 1: Determine if increases in steady state H2O2 within ovarian cancer cells enhance ascorbate cytotoxicity.
Aim 2: Assess the role of extracellular free iron within ascites in mediating ascorbate toxicity.
Aim 3: Test the validity of ascorbate as an adjuvant to IP chemotherapy.
Role: P.I.
Overlap: None

PROJECTS ENDED SINCE LAST REPORT:
R00 CA143229 (Hempel, Nadine) 3/21/2015-3/31/2016
NIH/NCI $111,580 DC/Yr
Contracting/Grants Officer: Viviana Knowles, Grants Management Specialist, National Cancer Institute, Viviana.knowles@nih.gov, 240-765-5157
Title: Mitochondrial Redox Control of Metastasis
Goals: This proposal will study the role of sub-lethal picomolar in mitochondrial ROS changes during metastasis and investigate their mechanisms of action as second messengers in pro-metastatic signaling events.
Specific Aims: Aim 1: To investigate the cellular site of action of mitochondrial ROS and their role in regulating oxidation, spatial distribution and protein fate of Protein Tyrosine Phosphatases (PTPs) involved in migratory signaling.
Aim 2: To further delineate the redox-regulation of p130cas.
Aim 3: To assess the consequences of enhanced intracellular ROS levels in an in vivo model of metastatic bladder cancer and the effectiveness of antioxidant therapy in this model.
Role: PI
Overlap: None

<u>OTHER SUPPORT - Kesterson, Joshua P.</u>

ACTIVE PROJECTS – Previously Reported
No Grant Number (Kesterson) August 2014 – Present 0 cal mths/unfunded effort
Veterans of Foreign Wars of the US Ladies $21,157 DC/Yr
Auxiliary Dept. of PA Research Grant for Ovarian Cancer Research

Contracting/ Grants Officer: Brenda M. Johnson, US Ladies Auxiliary Dept of PA, 4002 Fenton Avenue, Harrisburg, PA 17109

Title: Epithelial Ovarian Cancer Research

Goals: To be able to continue promising and exciting research endeavors in ovarian cancer research. In the absence of a sensitive screening strategy to diagnose Epithelial Ovarian Cancer (EOC) at an early stage, when survival is improved, efforts must be focused on developing new strategies to augment adjuvant cytotoxic chemotherapy to increase the interval between original diagnosis and recurrence.

Specific Aims: Aim 1: Understanding pathways by which ovarian cancer becomes resistant to standard chemotherapy
Aim 2: Means to target the OGF/OGFr axis to be susceptible to receptor agonist intervention with resultant inhibition of cell proliferation, angiogenesis and tumor growth

Role: Principal Investigator

Overlap: None

PROJECTS FUNDED SINCE LAST REPORT:

W81XWH-16-1-0117 (Hempel) 5/01/2016-4/30/2018 0.12 cal mths
U.S. Army Medical Research and Development Command $250,000 DC/Yr
Contracting/Grants Officer: Joshua Disbennett, Grant Specialist, Grants Administration Office, 301-619-7349, Grants Officer: Joshua.L.Disbennett.civ@mail.mil

Title: Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction

Goals: The goal of our study is to identify the clinical relevance and mechanism leading to mitochondrial dysfunction in ovarian cancer, and identify how this influences chemoresistance and ovarian cancer etiology.

Specific Aims: Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC.
Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on ovarian cancer mitochondrial function, metabolism and chemoresistance.
Aim 3: To investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs.

Role: Co-Investigator

Overlap: *This is the project for which the progress report is being submitted.*

Pilot Study (Hempel, Nadine) 4/1/2017-3/31/2018 0.12 cal mths
Marsha Rivkin Center for Ovarian Cancer Research $75,000 DC/Yr
Contracting/Grants Officer: Kiran Dhillon, PhD, Director of Scientific Programs, Rivkin Center for Ovarian Cancer, (206) 215-2964, Kiran.Dhillon@swedish.org.

Title: Pre-clinical Investigation of High Dose Ascorbate IP Therapy

Goals: Using a combination of patient-derived samples, cell culture models, and in vivo studies, we will test the feasibility of high dose ascorbate IP therapy and interrogate the molecular mechanisms of action as it pertains to ovarian cancer cells in the IP cavity tumor environment.

Specific Aims: Aim 1: Determine if increases in steady state H2O2 within ovarian cancer cells enhance ascorbate cytotoxicity.
Aim 2: Assess the role of extracellular free iron within ascites in mediating ascorbate toxicity.
Aim 3: Test the validity of ascorbate as an adjuvant to IP chemotherapy.
Role: Co-Investigator
Overlap: None

PROJECTS ENDED SINCE LAST REPORT:
None

OTHER SUPPORT - Phaeton, Rebecca

ACTIVE – Previously Reported:
WU-17-231 Reproductive Scientist (Phaeton) 07/01/2014– 06/30/2017 0 cal mths
Development Program, March of Dimes/Supplemental Funding $10,000 DC
Contracting/ Grants Officer: Amanda Heflin, Washington University School of Medicine, St. Louis
Project Title: Radioimmunotherapy: Targeting E6 and E7 viral antigens of Human Papillomavirus Induced Cervical Cancer
Goals: The additional funding from this supplemental grant will be used to perform the studies of mice treated with 177 Lu labeled antibodies with assessment of toxicities of white blood cells, platelets, liver function tests and long term assessment of bone marrow evaluation.
Specific Aims: To assess dose limiting toxicities of treatment in vital organs and establishing iodistribution patters of most effective radiolanthanide noted in Aim 1 in our RSDP Seed Grant.
Role: PI
Overlap: None

PROJECTS FUNDED SINCE LAST REPORT:
W81XWH-16-1-0117 (Hempel, Nadine) 5/1/2016-4/30/2018 0.12 cal mths
U. S. Army Medical Research and Development Command $250,000 DC/Yr
Contracting/Grants Officer: Joshua Disbennett, Grant Specialist, Grants Administration Office, 301-619-7349, Joshua.L.Disbennett.civ@mail.mil
Title: Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction
Goals: The goal of our study is to identify the clinical relevance and mechanism leading to mitochondrial dysfunction in ovarian cancer, and identify how this influences chemoresistance and ovarian cancer etiology.
Specific Aims: Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC.
Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on ovarian cancer mitochondrial function, metabolism and chemoresistance.
Aim 3: To investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs.
Role: Co-Investigator

Overlap: *This is the project for which the progress report is being submitted.*

Pilot Study (Hempel, Nadine) 4/1/2017-3/31/2018 0.12 cal mths
Marsha Rivkin Center for Ovarian Cancer Research $75,000 DC/Yr
Contracting/Grants Officer: Kiran Dhillon, PhD, Director of Scientific Programs, Rivkin Center for Ovarian Cancer, (206) 215-2964, Kiran.Dhillon@swedish.org.
Title: Pre-clinical Investigation of High Dose Ascorbate IP Therapy
Goals: Using a combination of patient-derived samples, cell culture models, and in vivo studies, we will test the feasibility of high dose ascorbate IP therapy and interrogate the molecular mechanisms of action as it pertains to ovarian cancer cells in the IP cavity tumor environment.
Specific Aims: Aim 1: Determine if increases in steady state H2O2 within ovarian cancer cells enhance ascorbate cytotoxicity.
Aim 2: Assess the role of extracellular free iron within ascites in mediating ascorbate toxicity.
Aim 3: Test the validity of ascorbate as an adjuvant to IP chemotherapy.
Role: Co-Investigator
Overlap: None

Grant 193394
Genetech Gynecologic Cancer (Phaeton) 4/01/2017 - 3/31/2018 0.24 cal mths
Young Investigator Career Development Award $135,000
Foundation for Women's Cancer
Contracting/Grants Officer: Jennifer Ocampo-Martinez, MPA, Manager of Governance/ASO Associate Executive Director 312-235-4060 ext. 271, jennifer.ocampo@sgo.org
Project Title: Mechanisms of ion Channels Involvement in Antibody Therapy of HPV Positive Cancers
Goals: We hypothesize that this treatment modality will 1) down-regulate volume sensitive chloride channels of the cervical epithelial membrane and 2) reverse the negative effect on CRAC channel activation in antitumor T-cells.
Specific Aims: Aim 1: Elucidate mechanism of entry of mAbs through cervical epithelial cell membrane and quantify mechanisms of induced immunogenicity. Aim 2: Evaluate the changes in the cervical epithelial membrane; both functional and Cl^- currents changes of VRACs induced by as a direct effect of mAb therapy decreasing E6 and E7 oncoprotein expression. Aim 3: Determine CRAC channel expression, function and regulation on T-lymphocytes after mAb therapy against HPV.
Role: PI
Overlap: None

ObGyn Departmental (Phaeton) 10/01/2016-09/30/2018 0.12 cal mths
Internal Request for Application $18,000
Contracting/Grants Officer: Renata Topic, ObGyn Financial Administrator , 717-531-6285, rtopic@pennstatehealth.psu.edu
Project Title: Development of a Biomarker for Detection of HPV Related Cervical Cancer

Goals: This proposal seeks to further expand the current knowledge by showing a relationship between specific high-risk HPV genotype and miRNA expression signatures to define new biomarkers for cancer diagnosis, treatment, and to prognosticate recurrence patterns

Specific Aims: Aim 1- To test the hypothesis that circulating miRNAs exhibit a differential expression signature in response to concurrent chemoradiotherapy across a spectrum of patient samples.

Role: PI

Overlap: None

Association of Faculty and Friends (Phaeton) 07/01/2016 – 06/30/2017 Unfunded Effort
Translational Ovarian Cancer Research $5,092
Contracting/Grants Officer: Mimi Legro, AFF President, 717-503-0884, mblegro@gmail.com

Project Title: None

Goals: Funding is to support and promote medical education, research, clinical care and scholarships

Specific Aims: Translational Ovarian Cancer Research. This funding is designated specifically for Capital Equipment for the incubator and consumables and cell culture reagents.

Role: PI

Overlap: None

PROJECTS ENDED SINCE LAST REPORT:

K12 2K12HD000849-28 (Moley/Phaeton) 7/01/2015-6/30/2016 9.0 cal mths
NIH/NICHD 100,000 DC
Reproductive Scientist Development Program (RSDP)
Contracting/Grants Officer: Amanda Heflin, Washington University School of Medicine, St. Louis

Project Title: Radioimmunotherapy: Targeting E6 and E7 viral antigens of Human Papillomavirus induced Cervical Cancer

Goals: The major goal of this project is to test the toxicity profile of the radiolabeled antibody to ensure safety and decreased side effect profile as this modality is moved toward Phase I clinical trials in patients with advanced, metastatic cervical cancer.

Specific Aims: Aim 1: To determine the most effective and least toxic radionuclide from the available beta-emitting radionuclides for the treatment of experimental cervical cancer.

Aim 2: To assess dose limiting toxicities of treatment in vital organs and establishing biodistribution patterns of most effective radiolanthanide noted in Aim 1.

Aim 3: To investigate the contribution of unlabeled (named) monoclonal antibodies toE6 and E7 to the efficacy of RIT.

Role: PI

Overlap: None

Reproductive Scientist Development Program (Phaeton) 07/01/2015 – 06/30/2016 0 cal mths
American Board of Obstetrics and Gynecology (ABOG) $25,000 DC

Supplemental Funding WU-16-55
Contracting/Grants Officer: Amanda Heflin, Washington University School of Medicine, St. Louis
Project Title: Radioimmunotherapy: Targeting E6 and E7 viral antigens of Human Papillomavirus induced Cervical Cancer
Goals: The additional funding from this supplemental grant will be used to perform the studies of mice treated with 177 Lu labeled antibodies with assessment of toxicities of white blood cells, platelets, liver function tests and long term assessment of bone marrow evaluation.
Specific Aims: Aim 2: To assess dose limiting toxicities of treatment in vital organs and establishing biodistribution patters of most effective radiolanthanide noted in Aim 1 in our RSDP Seed Grant.
Role: PI
Overlap: None

OTHER SUPPORT - Wang, Hong-Gang

ACTIVE – Previously reported:
5 P01 CA171983-04 (Wang, Hong-Gang) 9/10/2013-8/31/2018 2.28 cal mths
NIH/NCI prime, Subaward from University of Virginia $710,493 DC/Yr
Contracting/ Grants Officer: Robert Wilson, Cancer Center Senior Finance Generalist, UVA Cancer Center, Rw9u@hscmail.mcc.virginia.edu
Title: "Targeted Sphingolipid Metabolism for Treatment of AML" (Kester/Loughran/Wang)
Goals: The Program's broad-long-term objective is to develop new targeted therapeutics for acute myelogenous leukemia (AML).
Specific Aims: Aim 1: Engineer, characterize and optimize novel lipomimetic- or small molecule-based therapeutics for AML.
Aim 2: Validate the efficacy and toxicology of sphingolipid-targeted therapeutics in vivo using murine leukemia stem cells models.
Aim 3: Define the role of altered sphingofipid metabolism in cell survival, apoptosis, autophagy, and drug resistance in AML.
Role: MPI, Entire Project; Leader, Project 3; Co-Investigator, Project 1 & Core C
Overlap: None

PROJECTS FUNDED SINCE LAST REPORT:
W81XWH-16-1-0117 (Hempel, Nadine) 5/1/2016-4/30/2018 0.12 cal mths
U. S. Army Medical Research and Development Command $250,000 DC/Yr
Contracting/Grants Officer: Joshua Disbennett, Grant Specialist, Grants Administration Office, 301-619-7349, Joshua.L.Disbennett.civ@mail.mil
Title: Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction
Goals: The goal of our study is to identify the clinical relevance and mechanism leading to mitochondrial dysfunction in ovarian cancer, and identify how this influences chemoresistance and ovarian cancer etiology.
Specific Aims: Aim 1: To establish clinical significance of mitochondrial dysfunction in EOC.
Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant

expression on ovarian cancer mitochondrial function, metabolism and chemoresistance.
Aim 3: To investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs.

Role: Co-Investigator
Overlap: *This is the project for which the progress report is being submitted.*

1 R01 GM117014-01 (Miller, Barbara A.) 5/1/2016-4/30/2020 0.24 cal mths
NIH/NIGMS $198,000 DC/Yr
Contracting/Grants Officer: Director, Center for Scientific Review, National Institutes of Health, 6701 Rockledge Drive MSC 7768, Bethesda MD 20892-7768
Title: TRPM2, Mitochondria, and Cell Survival
Goals: This research will investigate whether TRPM2-L sustains cell proliferation and protects viability through moderate Ca2+ influx, which mediates HIF-1/2α expression, maintains mitochondrial function, and reduces production of reactive oxidant species.
Specific Aims: Aim 1: Is cell proliferation or viability reduced by inhibition of TRPM2 mediated Ca2+ influx? We will determine the role of TRPM2-mediated Ca2+ influx in modulation of cell viability, in vivo tumor formation, doxorubicin sensitivity, and ROS generation using TRPM2 loss and gain of function mutants.
Aim 2: Does TRPM2-mediated Ca2+ influx modulate mitochondrial function in neuroblastoma? Mitochondrial function including mitochondrial membrane potential, Ca2+ uptake, ATP production, and expression of BNIP3 and NDUFA4L2 are significantly reduced in cells expressing TRPM2-S, and ROS production is increased.
Role: Co-Investigator
Overlap: None

PROJECTS ENDED SINCE LAST REPORT:

5 R01 CA082197-16 (Wang, Hong-Gang) 6/1/2011-3/31/2017 1.92 cal mths
NIH/NCI $161,191 DC/Yr
Contracting/Grants Officer: Viviana Knowles, Grants Management Specialist, Office of Grants Administration, National Cancer Institute, NIH, EPS, 6120 Executive Blvd., Suite 243, Rockville, MD 20892-7150, weissh@mail.nih.gov
Title: Apoptosis Mechanisms and Human Cancer
Goals: The goal of this project is to elucidate the molecular mechanisms that control Bax activation, apoptosis, and tumorigenesis.
Specific Aims: Aim 1: To determine the role of Bif-1 in Bax oligomerization and insertion into OMM, cytochrome c release, and apoptosis.
Aim 2: To determine the molecular basis and functional significance of Bif-1 phosphorylation.
Aim 3: To explore the in vivo functions of Bif-1 by characterizing Bif-1 knockout mice
Role: P.I.
Overlap: None

R01 CA098472 (Wang, Hong-Gang) 10/1/2013-4/30/2017 0.60 cal mths
NIH prime / Subaward from University of Virginia $12,387 DC/Yr
Contracting/Grants Officer: Robert Wilson, Cancer Center Senior Finance Generalist, UVA Cancer Center, Rw9u@hscmail.mcc.virginia.edu
Title: Survival Mechanisms in Leukemic NK Cells
Goals: The long-term goal of this project is to develop better therapeutics for NK-LGL leukemia which is an incurable illness.
Specific Aims: Competing Renewal Aims:
Aim 1: To test the hypothesis that S1PR5 signaling mediates survival of leukemic NK cells
Aim 2: to test the hypothesis that FTY720 induces both apoptosis and autophagy in leukemic NK cells by targeting sphingolipid signaling.
Role: Consortium P.I.
Overlap: None

(Wang, Hong-Gang) 12/31/2014-12/31/2016 1.20 cal mths
Hyundai Hope on Wheels $125,000 DC/Yr
Contracting/ Grants Officer: Zafar J Brooks, 10550 Talbert Ave, Fountain Valley, CA 92708, info@hopeonwheels.org, 717-965-3584
Title: Switching autophagy to apoptosis for therapeutic benefit in pediatric acute myeloid leukemia
Goals: In this project, we will employ a number of innovative technologies, FDA-approved drugs, and humanized animal models to test this highly novel and innovative concept in pediatric AML.
Specific Aims: Aim 1: Define the mechanisms underlying the enhanced efficacy between HDACi and inhibitors of autophagic flux in pediatric AML cells.
Aim 2: Evaluate the efficacy and mechanism of action of HDACi combined with inhibitors of autophagic flux in xenograft mouse models of pediatric AML.
Role: P.I.
Overlap: None

OTHER SUPPORT - Warrick, Joshua

ACTIVE PROJECTS – Previously Reported
None

PROJECTS FUNDED SINCE LAST REPORT:
W81XWH-16-1-0117 (Hempel, Nadine) 5/1/2016-4/30/2018 0.12 cal mths
U. S. Army Medical Research and Development Command $250,000 DC/Yr
Contracting/Grants Officer: Joshua Disbennett, Grant Specialist, Grants Administration Office, 301-619-7349, Joshua.L.Disbennett.civ@mail.mil
Title: Clinical Significance and Mechanistic Insights into Ovarian Cancer Mitochondrial Dysfunction

Goals: The goal of our study is to identify the clinical relevance and mechanism leading to mitochondrial dysfunction in ovarian cancer, and identify how this influences chemoresistance and ovarian cancer etiology.

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Aim 2: To elucidate the mechanistic consequences of Drp1 splice variant expression on ovarian cancer mitochondrial function, metabolism and chemoresistance.
Aim 3: To investigate alternate therapeutic strategies for chemoresistant mitochondria-deficient EOCs.

Role: Co-Investigator

Overlap: *This is the project for which the progress report is being submitted.*

(Warrick, Joshua, PI) 1/1/2016-12/31/2017 0 cal mths
PA Tobacco Settlement Fund (TSF) $37,500 DC/Yr
Contracting/Grants Officer: John Anthony, Coordinator, Research and Administrative Services, Penn State University, 110 Technology Center, University Park, PA, 16802, jta11@psu.edu

Title: Cancer Risk Stratification of Endometrial Hyperplasia by Next Generation Sequencing

Goals: Determine if cancer gene sequencing can distinguish between benign and premalignant endometrial hyperplasia.

Specific Aims: Aim 1: Perform a case-control study to determine if extended mutational analysis of endometrial hyperplasia is predictive of cancer risk.
Aim 2: establish the mutational landscape of endometrial hyperplasia.

Role: Principal investigator

Overlap: None

Research Scholar Grant (Stairs, Douglas, PI) 1/1/2017-12/31/2020 0.2 cal mths
American Cancer Society $660,000 DC/Entire Project
Contracting/Grants Officer: William C. Phelps, PhD, Extramural Grants Department, American Cancer Society, 250 Williams Street, Atlanta, GA 30303-1002, (404) 329-4360, William.Phelps@cancer.org

Title: Mechanisms of Squamous Cell Carcinoma Invasion

Goals: Mechanistically understand how p120ctn inactivation cooperates with EGFR overexpression to induce invasion via NFkB signaling.

Specific Aims: Aim 1: Identify how combined EGFR overexpression and p120ctn inactivation lead to cellular invasion via cancer cell autonomous activation of NFkB intrinsic signaling.
Aim 2: Determine the mechanism by which p120ctn inactivation *in vivo* results in a more aggressive cancer when combined with EGFR overexpression than p120ctn inactivation does alone.

Role: Co-investigator

Overlap: None

PROJECTS ENDED SINCE LAST REPORT:
None

What other organizations were involved as partners?

Nothing to Report (N/A)

8. SPECIAL REPORTING REQUIREMENTS

N/A

COLLABORATIVE AWARDS:

N/A

QUAD CHARTS:

N/A

9. APPENDICES:

- Abstracts and Posters:
    - Shin DH, Dier U, Timmins PF, Kesterson J, Phaeton R, Hempel N Mitochondrial dynamics and dysfunction in ovarian cancer. September 2016, Rivkin Center for Ovarian Cancer and AACR – Ovarian Cancer Research Symposium, Seattle WA.
    - Shin DH, Kim Yeon Soo, Dier Usawadee, Timmins PF, Yoon Yisang, Kesterson Joshua, Phaeton Rebecca and Hempel N. The role of mitochondria fission protein Drp1 in metastatic ovarian cancer. May 2016 The NHLBI/NIDDK Mitochondrial Biology Symposium, National Institutes of Health, Bethesda, Maryland.
- Review Article:
    - Hempel N, Trebak M. Crosstalk between calcium and reactive oxygen species signaling in cancer. *Cell calcium*. (2017) Jan 18 (in press)

NHLBI Symposium on Mitochondria, May 2016, Bethesda, MD

Abstract:

The role of mitochondria fission protein Drp1 in metastatic ovarian cancer

Dong-Hui Shin[1], Yeon Soo Kim[1], Usawadee Dier[2], Patrick F. Timmins[3], Yisang Yoon[4], Joshua Kesterson[5], Rebecca Phaeton[5] and Nadine Hempel[1]

[1]Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA, USA
[2]Nanobioscience Constellation, State University of New York Polytechnic Institute, Albany, NY, USA
[3]Women's Cancer Care Associates, Albany, NY, USA
[4]Department of Physiology, Medical College of Georgia, Georgia Regents University, Augusta, GA, USA
[5]Department of Gynecology and Obstetrics, Pennsylvania State University College of Medicine, Hershey, PA, USA

Background Ovarian cancer remains the most deadly gynecological malignancy. A large proportion of patients are diagnosed at late stage, when significant metastasis has occurred through the intra peritoneal (IP) cavity and this is characterized by high rates of relapse and chemoresistance. Mitochondrial dysfunction plays an important role in both tumor growth, metastasis and chemoresistance. On the contrary, functional mitochondria are integral to proper apoptosis induction and it is thought that mitochondrial dysfunction is one mechanism by which cancer cells evade killing by this pathway. We recently showed that the ovarian high-grade serous adenocarcinoma cell line OVCA420 has dysfunctional mitochondria. In addition, a subgroup of cancer cells derived from ascites fluid of patients diagnosed with late stage disease showed a similar phenotype. The cause of mitochondrial dysfunction has been attributed to a number of factors including deregulation in mitochondrial fission and fusion dynamics. Here we investigated the role of different Drp1 variants in regulation of mitochondrial dynamics and function in ovarian cancer.

Methods Mitochondrial function of ovarian cancer cell lines and cells derived from ovarian cancer patients ascites fluid was assessed using Seahorse extracellular flux analysis. Mitotracker staining was used to analyze mitochondria morphology and expression levels of mitochondria fission related protein Drp1 determined using immunoblotting. The gene expression levels of cancer stem cell (CSC) markers in OVCA 420 cellular spheroid aggregates were assessed using Human Stem Cell RT2 profiler PCR Array.

Results We found that OVCA420 cells and one of 4 patient derived samples showed very low basal oxygen consumption rate and a lack in respiratory reserve capacity compared to other ovarian cancer cell lines, while relying primarily on glycolysis and glutamate utilization. Further, these cells displayed highly disordered mitochondria morphology with areas of high Mitotracker aggregation. We identified that the expression of a low molecular weight variant of the mitochondria fission protein Drp1 (short Drp1) is highly expressed in ovarian cancer with mitochondrial dysfunction. Importantly, a subgroup of cancer cells from patients also had overexpression of this short Drp1. This suggests that short Drp1 may represent a dominant negative form that inhibits mitochondrial fission, inhibiting mitochondrial function, and altering tumor metabolism. In addition, we found that expression of short and full length Drp1 expression are dynamically regulated during different stages of ovarian cancer progression. We investigated the formation of cellular spheroid aggregates, as a model of cells detaching from the primary tumor into the IP cavity. We found that short Drp1 expression is decreased and full length Drp1 protein is increased in spheroid aggregates. In addition, CSC-associated transcription factors Oct4 and Nanog were increased in spheroids. We are investigating if expression of short, potentially dominant-negative Drp1 is associated with a highly proliferative cellular phenotype, while full length active Drp1 is increased in CSC-enriched spheroids to maintain a healthy pool of mitochondria during anchorage independence.

Conclusion Dynamic Drp1 expression switching between the dominant-negative short and the full length Drp1 fission protein may be a novel way for cancer cells to regulate fission/fusion, mitochondrial quality control and metabolism.

The role of mitochondria fission protein Drp1 in metastatic ovarian cancer

Dong-Hui Shin[1], Yeon Soo Kim[1], Usawadee Dier[2], Patrick F. Timmins[3] and Nadine Hempel[1]

PENNSTATE HERSHEY College of Medicine

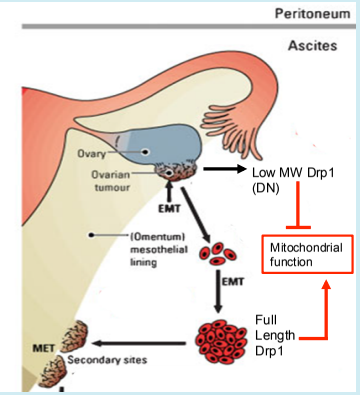
[1]Department of Pharmacology, Pennsylvania State University College of Medicine, Hershey, PA, USA
[2]Nanobioscience Constellation, State University of New York Polytechnic Institute, Albany, NY, USA
[3]Women's Cancer Care Associates, Albany Medical College, Albany NY, USA

Background

- Ovarian cancer remains the most deadly gynecological malignancy[1].
- A large proportion of patients are diagnosed at late stage, when significant metastasis has occurred through the peritoneal cavity and the disease is characterized by high rates of relapse and chemoresistance[2].
- Mitochondrial dysfunction is frequently observed in metastatic cancer cells[3], and may be related to metabolic adaptations of cancer cells, such as glycolysis.
- Functional mitochondria are integral to proper apoptosis induction and it is thought that mitochondria dysfunction may be one mechanism by which cancer cells evade killing by this pathway.
- The cause of mitochondria dysfunction has been attributed to a number of factors including deregulation in mitochondrial fission and fusion dynamics[4,5].

Hypothesis

Our hypothesis is that metastatic ovarian cancer cells dynamically regulate their mitochondrial function by alternate expression of Drp1 protein variants, depending on their stage of metastatic spread and energy demands.



Results

Bioenergetics Profiling identifies Ovarian Cancer cells with Mitochondrial Dysfunction

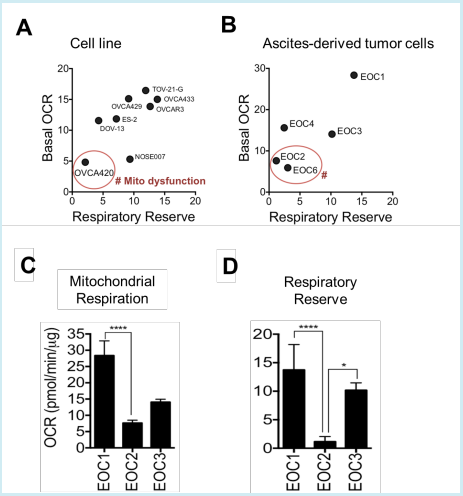


Figure 1. Extracellular flux analysis of ovarian cancer cell lines (A) and ascites-derived EOCs (B) identified a sub-set of mitochondria defective cells (#) with low oxygene consumption rate (OCR, C) and low Respiratory reserve capacity (D). *p < 0.05, ****p 0.001 (ANOVA)

Mitochondrial morphology of respiratory defective cells

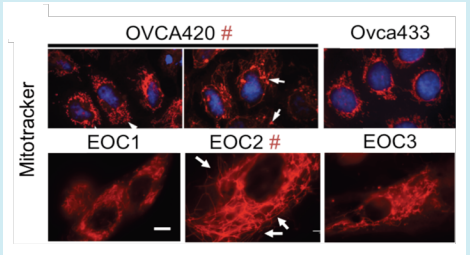


Figure 2. The cells with compromised mitochondria function (#) display aberration in mitochondria morphology with areas of hyperfusion.

Mitochondrial dysfunction is associated with expression of a low MW variant of the Fission Protein Drp1

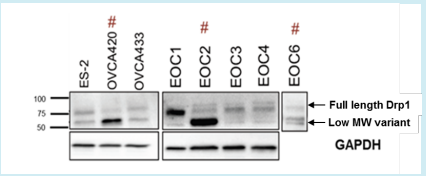


Figure 3. Drp1 protein expression in cell lines and ascites-derived samples indicated expression of a short Drp1 splice variant in mitochondria-defective cells (#).

Expression of Drp1 variants is dependent on anchorage and spheroid formation

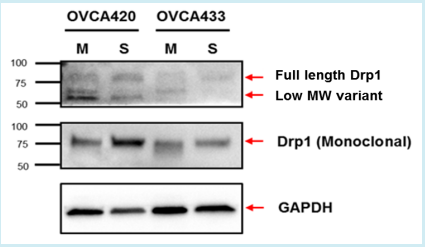
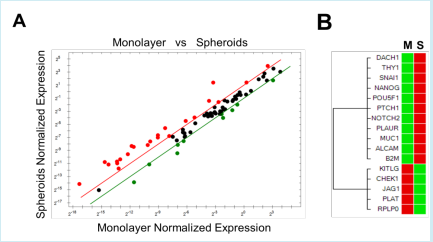


Figure 4. Expression levels of full length Drp1(~82KDa) increase and levels of low molecular variant (~60KDa) decrease when ovarian cancer cells are cultured as anchorage-independent spheroids (S) compared to attached monolayer cultures (M).

Anchorage independent spheroids are associated with full length Drp1 expression and Cancer Stem Cell Markers



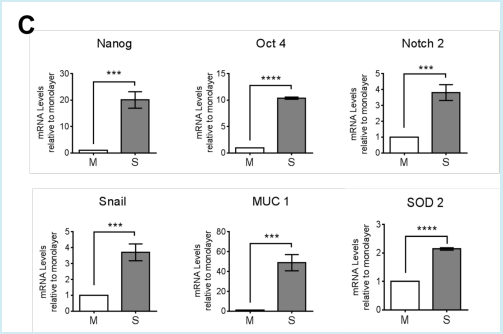


Figure 5. Gene expression levels of cancer stem cell (CSC) markers in OVCA 420 spheroid (S) compared with OVCA 420 monolayer (M). 84 genes involved in CSC were determined using Human Stem Cell RT2 profiler PCR Array (A). Green squares represent up-regulation and red squares represent down-regulation based on at least 2 fold difference (B). Up-regulated genes identified by PCR Array experiments were verified by an independent quantitative RT-PCR (C).

Conclusions

1. Mitochondrial dysfunction may be a hallmark of a subgroup of high grade serous adenocarcinomas.
2. During attachment expression of short Drp1 may act as a Dominant Negative (DN) to inhibit mitochondrial function and alter tumor metabolism towards glycolysis.
3. CSCs may have the ability to up-regulate full length Drp1 to maintain high mitochondria fidelity in anchorage independence.

Future Direction

1. Identification of Drp1 variants in Ovarian Cancer and correlation with mitochondrial dysfunction (RNA sequencing and Mass spectrometry), and patient outcome.
2. Mechanisms of low MW Drp1 expression and function as a Dominant Negative, and the dependence on the tumor microenvironment (ie nutrient availability)

References

1. Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2013. *CA: a cancer journal for clinicians* **63**, 11-30 (2013)
2. Davidson, B. Ovarian carcinoma and serous effusions. Changing views regarding tumor progression and review of current literature. *Analytical cellular pathology : the journal of the European Society for Analytical Cellular Pathology* **23**, 107-128 (2001).
3. Chen, E.I. Mitochondrial dysfunction and cancer metastasis. *Journal of bioenergetics and biomembranes* **44**, 619-622 (2012).
4. Boland, M.L., Chourasia, A.H. & Macleod, K.F. Mitochondrial dysfunction in cancer. *Frontiers in oncology* **3**, 292 (2013).
5. Twig, G. *et al.* Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *The EMBO journal* **27**, 433-446 (2008).

Funding: NIH/NCI: R00CA143229

Rivkin & AACR Ovarian Cancer research Symposium, September 2016, Seattle WA

Abstract:

Mitochondrial dynamics and dysfunction in ovarian cancer

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Altered mitochondrial function remains a key feature of many tumor cells and drives pathways such gene expression, metabolic and stress responses, cell cycle progression and resistance to apoptosis. Many chemotherapeutics activate programmed cell death and it is thought that mitochondrial dysfunction may be one mechanism by which cancer cells evade killing by these compounds. Screening the metabolic profiles of ovarian cancer cell lines and patient ascites-derived tumor cells reveals that ovarian cancers fall into unique bioenergetic subgroups. For example, Ovarian Clear Cell Carcinomas (OCCC) display high oxygen consumption rate and glycolytic flux compared to the more common high grade serous adenocarcinoma (HGSA) subtype. In addition, we show that a portion of HGSAs have severe mitochondrial dysfunction, that is marked by a decrease in mitochondrial respiration, a lack of response to the uncoupler FCCP and a concomitant reliance on alternate metabolic pathways. Moreover, this is accompanied by enhanced chemoresistance to Cisplatin and Taxol. The cause of mitochondrial dysfunction has been attributed to a number of factors, including deregulation in mitochondrial fission/fusion dynamics. Moreover, fission is an integral component of apoptotic and authophagy pathways. Interestingly, the observed HGSA mitochondrial dysfunction correlates with aberrant fusion/fission dynamics and expression of a low molecular weight variant of the mitochondrial fission protein Drp1. The potential significance of Drp1 in ovarian cancer etiology is highlighted by TCGA data, where more than 15% of HGSA samples display significant increases in Drp1 mRNA levels, and associated amplification of the Drp1 gene *DNM1L*. Whether this represents the shorter, potentially dominant-negative Drp1 variant identified in our work is currently under active investigation. Our data suggest that compromised mitochondrial function and fission/fusion dynamics may be a hallmark of a previously unidentified subgroup of highly chemoresistant EOCs and that this is associated with aberrant expression of the fission protein Drp1. Studies are underway to identify the molecular identity and regulation of short Drp1, and the mechanistic links to alterations in fission, metabolic switching and chemoresistance.

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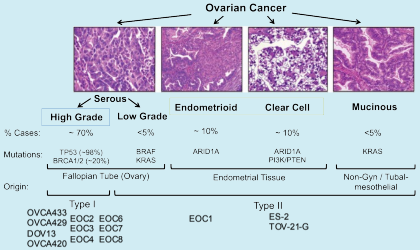
Abstract

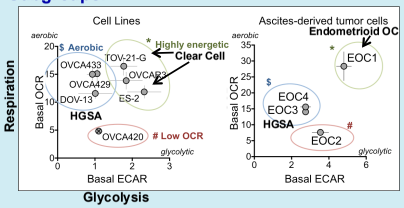
Altered mitochondrial function remains a key feature of many tumor cells and drives pathways such gene expression, metabolic and stress responses, cell cycle progression and resistance to apoptosis. Many chemotherapeutics activate programmed cell death and it is thought that mitochondrial dysfunction may be one mechanism by which cancer cells evade killing by these compounds. Screening the metabolic profiles of ovarian cancer cell lines and patient ascites-derived tumor cells reveals that ovarian cancers fall into unique bioenergetic subgroups. For example, Ovarian Clear Cell Carcinomas (OCCC) display high oxygen consumption rate and glycolytic flux compared to the more common high grade serous adenocarcinoma (HGSA) subtype. In addition, we show that a portion of HGSAs have severe mitochondrial dysfunction, that is marked by a decrease in mitochondrial respiration, a lack of response to the uncoupler FCCP and a concomitant reliance on alternate metabolic pathways. Moreover, this is accompanied by enhanced chemoresistance to Cisplatin and Taxol. The cause of mitochondrial dysfunction has been attributed to a number of factors, including deregulation in mitochondrial fission/fusion dynamics. Moreover, fission is an integral component of apoptotic and autophagy pathways. Interestingly, the observed HGSA mitochondrial dysfunction correlates with aberrant fusion/fission dynamics and expression of a low molecular weight variant of the mitochondrial fission protein Drp1. The potential significance of Drp1 in ovarian cancer etiology is highlighted by TCGA data, where more than 15% of HGSA samples display significant increases in Drp1 mRNA levels, and associated amplification of the Drp1 gene *DNM1L*. Whether this represents the shorter, potentially dominant-negative Drp1 variant identified in our work is currently under active investigation. Our data suggest that compromised mitochondrial function and fission/fusion dynamics may be a hallmark of a previously unidentified subgroup of highly chemoresistant EOCs and that this is associated with aberrant expression of the fission protein Drp1. Studies are underway to identify the molecular identity and regulation of short Drp1, and the mechanistic links to alterations in fission, metabolic switching and chemoresistance.



Ovarian Cancer histological subtypes (modified from Rescigno *et al.*, 2013). Cell lines and patient ascites-derived tumor cells (EOC) used in the study are listed below their classification. Ascites were obtained from stage III-IV ovarian cancer patients (SUNY / Penn State Hershey IRB approved study). Ovarian cancer cells were isolated and cultured, as previously described (Shepherd *et al.*, 2006 Nat Protoc;1:2643).

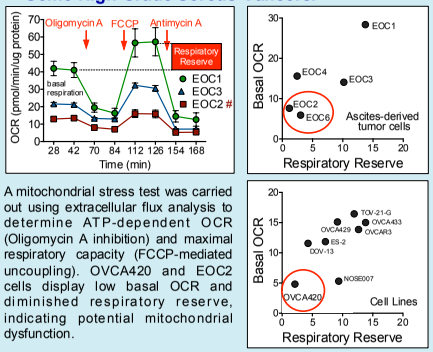
Results

1. Ovarian Cancers Fall Into Unique Bioenergetic Subgroups.



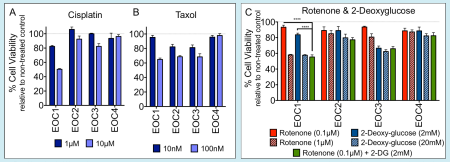
Bioenergetics profiles were obtained by assessing Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) using a Seahorse Extracellular Flux analyzer (Dier *et al.*, 2014, PLoS ONE, 9:e98479). Ascites-derived endometrioid ovarian cancer cells (EOC1) and Clear Cell Carcinoma cell lines (TOV-21-G, ES-2) display a highly energetic phenotype (*; high glycolysis-ECAR / high respiration - OCR). OVCA420 and EOC2, both high grade serous adenocarcinomas (HGSA) had very low basal OCR, indicative of low mitochondrial respiration (#).

2. Mitochondrial Dysfunction Is A Phenotype Of Some High Grade Serous Cancers.



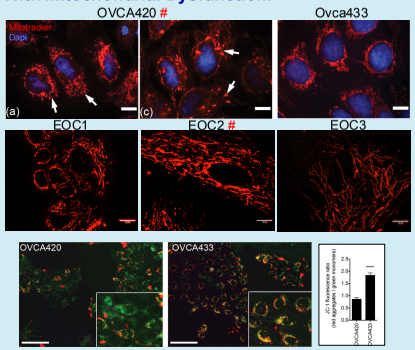
A mitochondrial stress test was carried out using extracellular flux analysis to determine ATP-dependent OCR (Oligomycin A inhibition) and maximal respiratory capacity (FCCP-mediated uncoupling). OVCA420 and EOC2 cells display low basal OCR and diminished respiratory reserve, indicating potential mitochondrial dysfunction.

3. Correlation Of Bioenergetics With Chemoresistance And Response To Metabolic Inhibitors.



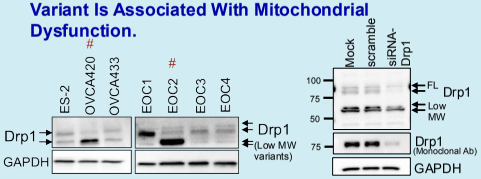
Cell viability in response to compounds was assessed using crystal violet staining, following 72 hrs of treatment. n=6, ****p<0.001 (ANOVA) Combination treatment of mitochondrial poisoning and glycolysis inhibition effectively target highly energetic ovarian cancer cells.

4. Aberrant Mitochondrial Fission/Fusion Correlates With Mitochondrial Dysfunction.



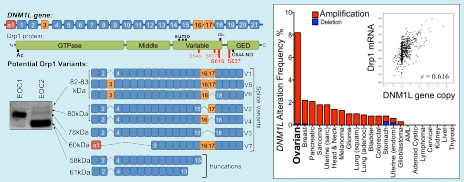
Mitotracker CMX ROS staining was used to visualize mitochondrial morphology. Arrows show areas of irregular mitochondrial morphology and large globular aggregates in OVCA420 and EOC2 cells (#). OVCA420 cells display a loss of mitochondrial membrane potential (JC-1 dye).

5. Expression Of A Low Molecular Weight Drp1 Variant Is Associated With Mitochondrial Dysfunction.



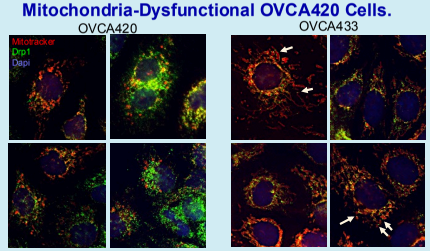
Cells with irregular mitochondrial fission (OVCA420 & EOC2 #) display enhanced expression of a low MW (~55-60kDa) variant of the fission protein Drp1. Transfection of an siRNA smartPool confirms that the observed low MW variant is Drp1.

6. Potential *DNM1L*/Drp1 Variants And *DNM1L* Copy Number Variation / Expression In Ovarian Cancer.



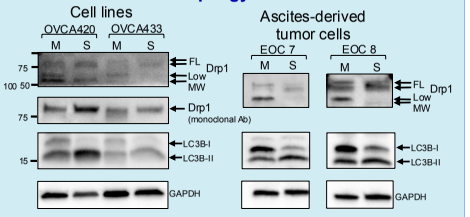
The *DNM1L* gene encodes for Drp1. Splice variants depicted are based on sequences from GenBank. RNA sequencing and mass spec analysis are underway to identify the observed variants in EOC samples and Ovarian cancer cell lines. Interestingly, Ovarian Cancer has a high frequency of *DNM1L* gene amplifications compared to other cancers, and expression of Drp1 correlates with *DNM1L* copy number (log2; TCGA derived data, CBioportal). We are investigating if certain splice and truncation variants represent dominant negative forms of Drp1 in ovarian cancer.

7. Drp1 Is Miss-localized To The Cytoplasm In Mitochondria-Dysfunctional OVCA420 Cells.



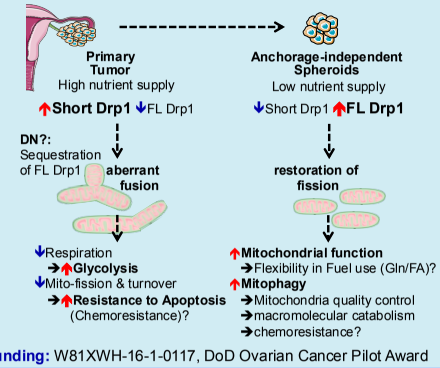
Immunofluorescence staining of Drp1 reveals distinct punctate localization at mitochondrial fission sites in OVCA433 cells (arrows), while Drp1 is distributed in the cytosol in OVCA420 cells.

8. Expression Of Low MW And Full Length Drp1 Is Dynamic Depending On Culture Conditions And Correlates With Autophagy.



Cells grown as anchorage-independent spheroids loose expression of low MW Drp1 and increase expression of full length Drp1. This switch is associated with increased LC3B-II levels, and indication of increased auto/mitophagy in spheroids.

Model



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Crosstalk between calcium and reactive oxygen species signaling in cancer

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ABSTRACT

The interplay between Ca^{2+} and reactive oxygen species (ROS) signaling pathways is well established, with reciprocal regulation occurring at a number of subcellular locations. Many Ca^{2+} channels at the cell surface and intracellular organelles, including the endoplasmic reticulum and mitochondria are regulated by redox modifications. In turn, Ca^{2+} signaling can influence the cellular generation of ROS, from sources such as NADPH oxidases and mitochondria. This relationship has been explored in great depth during the process of apoptosis, where surges of Ca^{2+} and ROS are important mediators of cell death. More recently, coordinated and localized Ca^{2+} and ROS transients appear to play a major role in a vast variety of pro-survival signaling pathways that may be crucial for both physiological and pathophysiological functions. While much work is required to firmly establish this Ca^{2+}-ROS relationship in cancer, existing evidence from other disease models suggests this crosstalk is likely of significant importance in tumorigenesis. In this review, we describe the regulation of Ca^{2+} channels and transporters by oxidants and discuss the potential consequences of the ROS-Ca^{2+} interplay in tumor cells.

Contents

Abbreviations: AMPK, 5′ adenosine monophosphate activated protein kinase; ASK1, apoptosis signal-regulating kinase 1; Atg, autophagy Related Cysteine Peptidase; BIRD-2, Bcl-2/IP3R disrupter; CaM, calmodulin; CaMKII, Ca^{2+}/Calmodulin-dependent kinase II; Cat, Catalase; CRAC, Ca^{2+} release activated Ca^{2+}; DAG, diacylglycerol; ER, Endoplasmic Reticulum; Ero, ER oxidoreductase; ETC, Electron Transport Chain; GPCR, G-protein coupled receptor; GPx, Glutahione Peroxidase; GR, glutathione reductase; Grx, glutaredoxin; GSH, Glutathione; HK, Hexokinase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; HIF, Hypoxia Inducible Factor; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; Lamp1, lysosomal-associated membrane protein 1; LC3-II, Microtubule-associated proteins 1A/1B light chain 3B; MAM, mitochondria associated membrane; MCUR1, MCU Regulator 1; MICU, Mitochondrial Ca^{2+} Uptake; mPTP, mitochondria Permeability Transition Pore; mTOR, mechanistic target of rapamycin; NCLX, Na^+/Ca^{2+} Li^+ Exchanger; NCX, Na^+/Ca^{2+} exchanger; NAD, nicotinamide adenine dinucleotide; NO, •nitric oxide; NOS, Nitric Oxide Synthase; Nox, NADPH oxidase; O2, •−superoxide; •OH, hydroxyl radical; $ONOO^-$, peroxinitrite; PARP, poly(ADP-ribose) polymerases; PDH, Pyruvate dehydrogenase; PDI, protein disulfide isomerase; PERK, RNA-dependent protein kinase (PKR)-like ER kinase; PI3K, Phosphoinositide 3-kinase; PLC, phospholipase C; PKC, Protein kinase C; PTEN, phosphatase and tensin homolog; Prx, Peroxidase; PMCA, plasma membrane Ca^{2+} ATPase; RNS, Reactive nitrogen Species; ROS, reactive oxygen species; RNS, reactive nitrogen species; RyR, Ryanodine Receptor; SERCA, Sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOCE, store operated calcium entry; Sod, Superoxide dismutase; TFEB, transcription factor EB; TRP, Transient Receptor Potential; Trx, thioredoxin; VDAC, voltage-dependent anion channel.

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1. Introduction

The relationship between Calcium (Ca^{2+}) and reactive oxygen/nitrogen species (ROS/RNS) is well established and has been described in numerous disease models. Much of our knowledge has been gained from the cardiovascular system, where this interplay is an important aspect of pathophysiology, a prominent example being ischemia/reperfusion injury, where the Ca^{2+}- ROS interplay is involved in eliciting cell death [1]. Thus, apoptosis is one event where coordinated surges of ROS and Ca^{2+} have been observed and studied in great depth [2–4]. However, in addition to cell death, emerging evidence reveal that many diverse cellular signaling events are regulated by concomitant and localized increases in ROS and Ca^{2+} transients [5–8]. This Ca^{2+} – ROS interaction is evident by the fact that many regulators of Ca^{2+} signaling are redox modified, and reciprocally Ca^{2+} signaling is intricately involved in regulating ROS levels. Importantly, the subcellular location of Ca^{2+} stores and the sites of ROS production are closely linked, prominently the ER-mitochondrial interface and the plasma membrane [9,10].

Tight regulation of Ca^{2+} homeostasis lies at the center of cellular signaling. The type of signaling "output" is dependent on the duration, localization, amplitude and frequency of the Ca^{2+} signal [11,12]. Regulation of Ca^{2+} homeostasis is achieved by a number of ion channels, pumps and exchangers, found on both the cell surface and the organelles that act as primary intracellular Ca^{2+} stores. Similarly, subcellular regions of ROS/RNS production, such as the leading edge of migrating cells and the ER-mitochondrial interface, are emerging as hubs of signaling, and, as highlighted below, the type of reactive species and signal amplitudes influence the consequential signaling events and cellular responses [13–15]. While many studies have examined the redox control of Ca^{2+} homeostasis, relatively few studies have investigated this connection specifically as it pertains to carcinogenesis or metastatic progression. This may in part be due to the fact that the role of Ca^{2+} signaling in cancer is a relatively new field and that Ca^{2+} signaling mechanisms are complex and do not adhere to a "one size fits all" paradigm in cancer cells [16]. Much like changes in redox balance, this appears to be context and cancer type specific. Underlying genomic differences between tumor types, cellular heterogeneity of individual tumors, and the contribution of the tumor microenvironment likely contribute to this variability. Nevertheless, a number of studies have demonstrated that increased cytosolic Ca^{2+} is involved in processes such as proliferation, migration, invasion, and anchorage independent survival, clearly demonstrating that Ca^{2+} signaling is important in cancer progression [16–19]. In the present review, we focus on the interplay between Ca^{2+} and ROS in cancer, highlighting some of the discoveries pertaining to the redox regulation of Ca^{2+} transport mechanisms, and how Ca^{2+} signaling pathways in turn may regulate the cellular redox environment. Although much work is still required to firmly establish this relationship in different cancer types, two themes can be inferred from existing literature. 1) Coordinated ROS and Ca^{2+} surges are required for apoptosis initiation at the mitochondrial-Endoplasmic Reticulum (ER) interface, with evidence suggesting that this interplay is altered in cancer cells to enhance apoptosis resistance. 2) Localized, sub-lethal changes in both ROS and Ca^{2+} levels fine-tune signaling cascades that maintain proliferative and metastatic signals (Fig. 1).

2. Oxidants – the importance of what, where and how much

2.1. What and where?

The terms reactive oxygen species (ROS) and reactive nitrogen species (RNS), are often loosely used to describe a group of very different molecular species that vary in reactivity, half-life, site of production and detoxification reactions (Fig. 2). These oxidants can be either free radicals (containing an unpaired electron), such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), or non-radical oxidants, including hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl, primarily in neutrophils) and peroxinitrite ($ONOO^-$), the latter being generated in the presence of $O_2^{\bullet -}$ and nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ is produced by Nitric oxide synthase, of which two isoforms (nNOS/NOS1 and eNOS/NOS3) are regulated by Ca^{2+} in a calmodulin-dependent manner [20]. It should be noted that these species vary widely in their half-life, reactivity and diffusion rates, and their role on macromolecular oxidation is dependent on amounts and sites of generation, as well as the rate of oxidation and abundance of target moieties [21]. Moreover, the reaction with target molecules, such as other ROS, lipids, proteins and DNA, is dependent on the redox environment of the cell. For example, high abundance of reduced glutathione and fast reaction with more readily oxidized proteins, such as peroxiredoxins, may result in "scavenging" of the oxidant species before these are able to reach their target (Fig. 2) [21–23]. The relatively high reactivity of some oxidants limits their diffusion and role as true signaling molecules. This includes the highly reactive $^{\bullet}OH$ ($T_{1/2}$ 10^{-6}-10^{-9}sec). Similarly, $O_2^{\bullet -}$ has a half-life of micro to milli seconds, depending on its environment and interactions with cellular and extracellular components such as $NO^{\bullet}$, transition metals and ascorbic acid; while H_2O_2 has a half life in the order of seconds [24,25].

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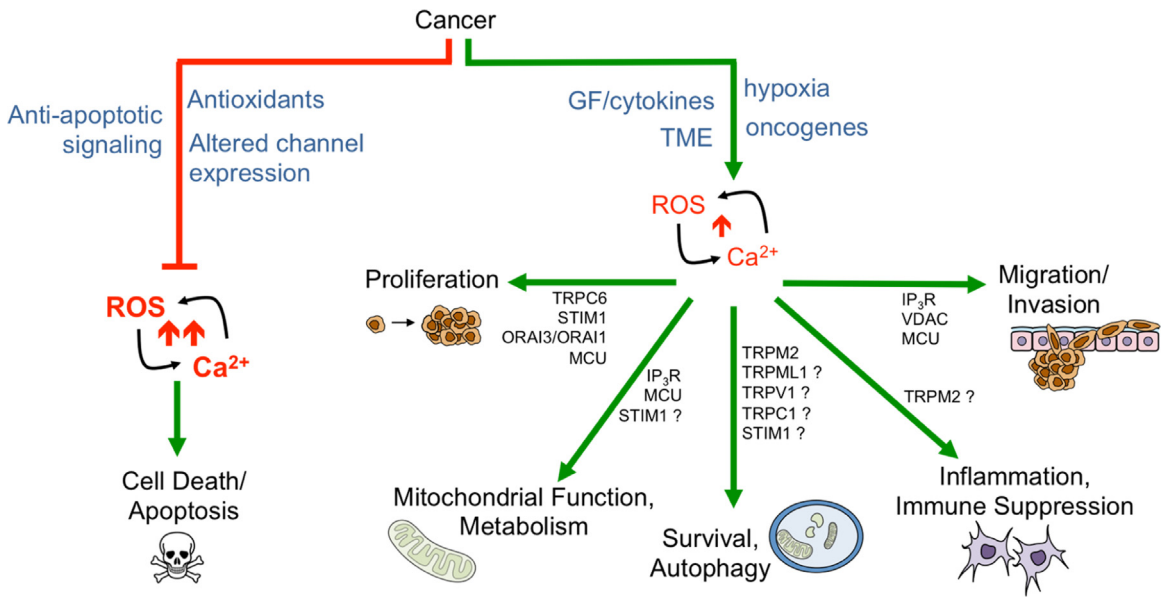

Fig. 1. Cancer cells take advantage and manipulate the ROS-Ca^{2+} interplay in two ways: 1) by inhibiting large ROS-Ca^{2+} surges that mediate apoptosis (red pathway). Inhibition of Ca^{2+} ER-mitochondrial transfer by inhibition of receptors and channels such as IP_3R and VDAC and subsequent suppression of mitochondrial ROS production are pathways by which cancer cells can evade apoptosis (Fig. 9); and 2) by promoting pro-tumorigenic signaling pathways in response to sublethal changes in ROS/Ca^{2+} levels. Alterations in ROS and Ca^{2+} levels are often consequences of signaling from Growth factors and cytokines, oncogene expression, and changes in the Tumor microenvironment (TME), including presence of tumor associated fibroblast and macrophages, hypoxia and nutrient stress. ROS are able to directly oxidize or indirectly manipulate activity of Ca^{2+} channels, pumps and regulators, including plasma membrane and ER and mitochondria localized channels (Fig. 3), while Ca^{2+} signals are known modulators of several ROS generating systems including NADPH oxidases (Nox), NO synthase (NOS) and the mitochondria (Fig. 2). In this review we will highlight examples of this crosstalk and how this may relate to pro-tumorigenic signaling. Question marks indicate Ca^{2+} regulators that have been implicated in driving cellular responses in a ROS dependent manner in other cell models, besides cancer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Nox Family Members.

Nox Isoform	Major Cell Type Expression	Cellular Localization	Activation
Nox1	Colon, Vasculature	Plasma Membrane/Caveolae	Rac/$p22^{phox}$/Noxa1/Noxo1
Nox2	Phagocytes	Plasma Membrane/Vesicles	Rac/$p22^{phox}$/$p47^{phox}$/$p40^{phox}$/$p67^{phox}$
Nox3	Inner Ear	Plasma Membrane	Rac/$p22^{phox}$/Noxa1/Noxo1
Nox4	Kidney, Vasculature	Plasma Membrane/ER/Mitochondria	$p22^{phox}$
Nox5	Lymphoid Tissues	Plasma Membrane	Ca^{2+}
Duox1/2	Thyroid	Plasma Membrane	Ca^{2+}

Oxidants are produced at a number of cellular locations that are important hubs for intracellular Ca^{2+} regulation, including the plasma membrane- endoplasmic reticulum (ER) junctions, and the interface between the ER and the mitochondria (Fig. 2). The primary oxidant produced within cells is $O_2^{\bullet -}$, which is generated enzymatically by membrane-bound NADPH oxidases (Nox) or through electron leakage of the electron transport chain (ETC) within mitochondria [10,26,27]. Nox enzymes (Table 1) utilize NADPH as the electron donor to generate $O_2^{\bullet -}$ from O_2, which is rapidly converted to H_2O_2. Although Nox-derived $O_2^{\bullet -}$ has been implicated in numerous studies in driving redox modifications within the cell, it is unlikely that this short lived oxidant is able to diffuse the plasma membrane and enter the cell. Rather, it likely reacts quickly with extracellular components such as ascorbic acid, or is dismuted to H_2O_2. H_2O_2 is able to traverse the plasma membrane, likely through aquaporins [28]. Nox 4 is thought to be able to directly generate H_2O_2 [29]. Nox enzymes form signaling complexes at cell membranes, and their regulation by growth factor and cytokine receptors, small GTPases, and second messengers, such as Ca^{2+}, illustrates that specific, localized activation of ROS production is an important aspect of cellular signaling [8,10].

Nox2, was the first of seven Nox family members to be characterized, and is primarily involved in regulating ROS surges in response to cytokine stimuli in phagocytic cells. Nox enzymes of non-phagocytic cells have different functions based on their compartmentalization and regulation, and are implicated in a number of pathophysiological conditions, including cancer. For example, Nox1-mediated ROS production is necessary for invadipodia formation to drive tumor cell migration [30,31]. Nox1-3 enzymes are regulated by the small GTPase Rac, which acts as a relay for Nox activation *via* a number of stimuli, including sheer stress, growth factors and lysophosphatidic acid (For further details on Nox family members and activation mechanisms we refer the reader to reviews by Brandes et al. [10] and Block and Gorin [32]). Further, cytokines and growth factors are able to induce Nox phosphorylation and activity through a variety of kinases and these mechanisms vary depending on the particular receptor ligand and Nox isoform involved. Nox1, 4 and 5 have specifically been implicated in ROS generation in cancer, and shown to be activated by receptor tyrosine kinases, G protein-coupled receptors (GPCRs) and oncogene signaling pathways [32]. Highlighting the evident interplay between ROS and Ca^{2+}, Nox5, Duox1 and Duox2 are regulated by Ca^{2+}, either directly through interactions with Nox-EF hands, or indirectly by calmodulin and protein Kinase C (PKC) [33–37]. Ca^{2+} channels may also directly interact with Nox at the cell surface to coordinate ROS-Ca^{2+} signaling. An example being the proposed interaction of the diacylglycerol (DAG)-sensitive transient receptor potential canonical 6 (TRPC6) cation channel with Nox2 in lipid rafts of podocytes [38,39]. In this example, the authors proposed that TRPC6-associated Nox2 is activated by DAG, and that subse-

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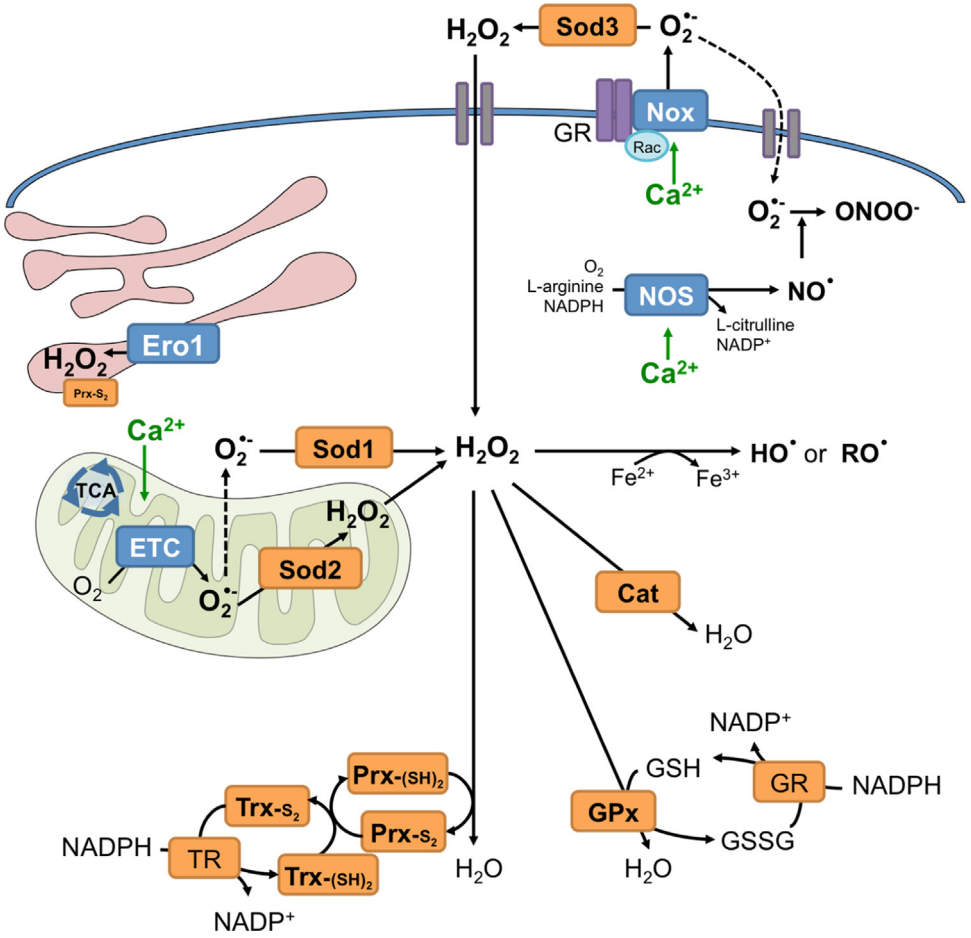

Fig. 2. Examples of common cellular reactive oxygen and nitrogen species. Ca^{2+} is involved in directly regulating some ROS/RNS "generators" (blue boxes), including NADPH oxidase isoforms Nox5, Duox1 and Duox2, and nitric oxide synthase NOS1 and NOS3. Ca^{2+} is important for the regulation of Tricarboxylic Acid (TCA) cycle and electron transport chain (ETC) enzymes and may in turn drive superoxide $O_2^{\bullet-}$ production in the mitochondria. The short-lived $O_2^{\bullet-}$ is likely unable to diffuse far from its site of production, but rather rapidly converted to H_2O_2. H_2O_2 can further react with iron to produce highly reactive hydroxyl radical or "scavenged" by Peroxiredoxins (Prx), Glutahtione (GSH) and catalse (Cat; orange boxes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quent Nox2-produced ROS further contribute to TRPC6 activation. As discussed below, the amount and duration of ROS/Ca^{2+} signals are important determinants for the consequential outcome of tumor cells. Therefore, the expression of ROS generating enzymes, such as Nox, can greatly influence different cellular responses. For example, small increases in Nox5 expression lead to proliferation of several types of cancer cell lines [32]. Conversely, when Nox5 levels reach a specific threshold of expression, $O_2^{\bullet-}$ production in response to Ca^{2+} stimulation can reach toxic levels leading to ROS-mediated apoptosis [40].

Mitochondria are major sources of cellular oxidants [10,26,27,41], and are recognized reservoirs of Ca^{2+} containing specific channels and transporters that tightly regulate mitochondrial matrix Ca^{2+} homeostasis [42,43]. Although it was initially thought that surges of ROS stemming from mitochondria are primarily involved in the process of apoptosis, recent evidence suggest that mitochondrial ROS production contributes to processes such as autophagy and pro-tumorigenic redox-signaling [44–47]. During respiration, electron leakage contributes to $O_2^{\bullet-}$ formation from oxygen (O_2). Complex I and III of the ETC have been primarily implicated in this process, leading to $O_2^{\bullet-}$ production into both the intermembrane space (complex III) and matrix (complex I & III) [26,27]. Whether or not $O_2^{\bullet-}$ is stable enough to elicit cellular signaling outside the mitochondria is still debated. Most likely, it is rapidly converted to the less reactive, more stable and readily diffusible H_2O_2, either spontaneously or by manganese superoxide dismutases Sod2 in the matrix and Sod1 in the intermembrane space. Due to the much longer half life of H_2O_2 (seconds), H_2O_2 may therefore represent a more likely candidate as a "redox second messenger", rather than $O_2^{\bullet-}$. Major drivers of mitochondrial ROS production during normal and patho-physiological conditions include alterations in mitochondrial electron transport chain complex function, hypoxia and hyperoxia, cytokine and oncogene signaling, including TNFα and the Phosphoinositide 3-kinase (PI3K) – Akt –target of rapamycin (mTOR) pathway [48–51]. Moreover, increased flux of Ca^{2+} into mitochondria is a driver of mitochondrial ROS production and an integral component of processes such as apoptosis, as discussed in some detail below [52,53].

The influx of Ca^{2+} into mitochondria occurs largely at domains termed mitochondria associated membranes (MAMs), where mitochondria are in close vicinity to the ER. The ER is a major intracellular Ca^{2+} store and is another source of cellular ROS. Protein folding, a primary function of the ER, is dependent on oxidative modification of cysteine thiols and subsequent disulfide bond formation. Therefore, the ER is a more oxidative environment than the cytoplasm and, as a consequence of the protein folding machinery, contributes to the production of H_2O_2 [54]. Disulfide bonds are exchanged between ER oxidoreductases (Ero1) and Protein disulfide iosmerases (PDIs), and these subsequently target proteins requiring disulfide bond formation for proper folding [54]. This concomitantly results in reverse shuttling of electrons from PDI

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to Ero1, and the reduction of molecular oxygen to H_2O_2 (Fig. 2) [55], however other pathways of H_2O_2 production in the ER which are Ero1-independent have been reported [56]. Disulfide formation within Peroxiredoxin 4 (Prx4) in the ER lumen is likely the direct target of ER-produced H_2O_2, and Prx4 has in turn been shown to transmit this redox signal (i.e. disulfide bond exchange) and to contribute to the protein folding machinery [57,58]. Another example of a redox sensor and ROS-producing protein localized at MAMs is p66shc. Under normal conditions, this protein is a RAS adaptor protein, but following pro-apoptotic stimuli, such as activation of apoptosis signal-regulating kinase 1 (ASK1), p66shc is translocated to MAMs, where it acts to further induce ROS production by interacting with cytochrome *c*. This interaction appears to enhance electron transfer to molecular oxygen and the generation of ROS [59].

Evolution has provided the cell with a sophisticated arsenal to prevent the accumulation oxidants (Fig. 2). $O_2^{\bullet -}$ is either spontaneously or enzymatically (*via* Sod) dismuted to H_2O_2. H_2O_2 is readily transported across cellular membranes [28,60], including the most likely through aquaporins in the plasma membrane and its levels are regulated by catalase within peroxisomes, glutathione peroxidases, and the peroxiredoxin/thioredoxin system throughout the cell. However, in the presence of transition metals (Fe^{2+} or Cu^{+}), H_2O_2 undergoes the Fenton reaction to yield highly reactive $^{\bullet}OH$. $^{\bullet}OH$ is the major oxidant responsible for DNA oxidation. Therefore, the reactivity and relative abundance of antioxidant scavengers greatly influence the consequential effects of individual oxidants. Studies interrogating the identity of specific oxidants regulating Ca^{2+} signaling proteins remain few.

The term ROS is often loosely and incorrectly used in the literature to explain a plethora of biological phenomena linked to changes in the redox status of cells [61]. Much of this can be attributed to our limitations in appropriate molecular tools, which are needed to adequately identify the species involved, quantitatively measure their relative amounts, and identify their sites of production, distribution and eventual site of action. While we will not dwell on the limitations of current methods, it should be mentioned that many commonly used redox sensors and ROS scavengers have limitations in sensitivity and accuracy. Moreover, the ability of some of these compounds to react with ROS and consequentially produce reactive species themselves further complicates their use. An example of this is the commonly referenced redox sensitive dye Dichlorodihydrofluorescein (DCF), which has specifically received much criticism for its use [62]. Although it is a useful screening tool, verification of data with appropriate scavenger controls and complementary methods, such as recombinant redox sensors and more sophisticated techniques including electron paramagnetic resonance (EPR) coupled with spin-trapping, are advised [7,63–65]. As with redox sensitive probes, another challenge in the field is the need for specific scavengers of reactive oxygen species in order to help identify roles of different oxidants. Many selectively designed scavengers have secondary effects and can independently affect the redox status of cells, either by acting as oxidants themselves or by activating transcription of antioxidant enzymes [62,66].

2.2. How much? – Redox stress versus redox signaling

Similar to Ca^{2+} signaling, the effects of oxidants are dependent on their relative levels and half-life. It is well known that differences in the frequency and amplitude of Ca^{2+} signals can have major consequences on eliciting diverse downstream cellular outputs [12]. Fine-tuned regulation of Ca^{2+} oscillations is thought to be one mechanism that accounts for the large variety of Ca^{2+}-mediated functions emanating from the same Ca^{2+} channel. An example is the difference in Ca^{2+} signals resulting from the ER-localized inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R). Sustained Ca^{2+} release from the ER leads to Ca^{2+} overload in mitochondria that sets off the apoptosis death cascade [67], while controlled oscillations initiate Ca^{2+} signals leading to numerous Ca^{2+} regulated signaling pathways, such as NFAT activation *via* calcineurin, an important transcription factor during proliferation [68,69]. Similarly, it is appreciated that large ROS surges, or "oxidative stress", are primarily associated with widespread oxidation of macromolecules and irreversible cellular damage, while sub-lethal changes contribute to redox signaling, as described in more detail below. In the case of immune responses, bolus doses of ROS are generated by phagocytic cells to initiate cell death of the pathogen and infected host cell [70]. In cancer, differences in the levels of ROS and their particular consequences are apparent at different stages of tumor progression. For instance, during tumor initiation/carcinogenesis, exogenous sources of ROS, such as radiation and chemical carcinogens, lead to oxidative damage of macromolecules, including DNA [71]. The resultant genomic instability and incorporation of mutations are well-known drivers of tumor suppressor inhibition and oncogene activation. However, tumor cells readily adapt to and are able to cope with oxidative stress. For example, tumor cells readily activate the Nrf2/Keap1 stress response pathway to increase expression of antioxidant enzymes [72]. This may also provide tumor cells with advantages when faced with changing tumor microenvironment during metastatic progression, including anchorage independent survival, hypoxia and nutrient stress [73,74]. Similarly, it has been shown that tumor cells can restrict Ca^{2+} influx from the extracellular milieu and inhibit sustained transfer of Ca^{2+} from the ER to the mitochondria to inhibit apoptosis [2,75–78]. Hence, large intrinsic surges of ROS and Ca^{2+} are generally avoided by tumor cells to prevent cell death.

More recently it has been appreciated that cancer cells inertly are able to adapt to redox stress during tumor progression, and may even thrive with an increased threshold of endogenously generated ROS, utilizing this for redox-mediated signaling [15,51]. Since cancer cells appear to operate under a higher cellular ROS steady state, there is a precedent to utilize this as an "Achilles Heel" for therapeutic targeting [79,80]. Many tumor cells are more susceptible to cell death in response to exogenous ROS, or ROS generating agents [40,81–83]. Hence, an increased cellular redox milieu may place tumor cells closer to the oxidative stress cytotoxic threshold in response to exogenous ROS.

Although the alterations in global redox status appear to be a phenotype of cancer cells, it is important to elucidate how this drives tumorigenesis and metastatic progression through Ca^{2+} signaling pathways. As evident from other cell model systems, coordinated, localized production of ROS sets up hubs of redox and Ca^{2+} signaling important for cellular function [84–86]. Like Ca^{2+} oscillations, or "sparks", similar observations of ROS flashes have been made with the advent of genetically engineered redox probes, including the redox-sensitive variant of GFP (roGFP) and HyPer [7,63,87]. For example, it has been suggested that ROS sparks at the plasma membrane leading edge are necessary for cell migration, and similar observations have been made for Ca^{2+} flickers. Studies on cardiomyocytes have shown that ROS and Ca^{2+} sparks are coordinated and are susceptible to stimuli, such as mechanical stretch [88,89]. Similarly, wounding of *C. elegans* skin, elicits a localized Ca^{2+} response that promotes localized mitochondrial ROS sparks, necessary for actin-mediated wound closure [86]. However, it is unknown if these events are similarly coordinated and mechanistically linked during cancer cell migration, an important facet to metastatic spread.

With our understanding that ROS are spatially and temporally regulated within cells and the appreciation that redox modifications of proteins are important regulatory mechanisms, redox

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signaling has received much attention over the past decades. A number of tumorigenic stimuli such as cytokines and growth factors can initiate $O_2^{\bullet-}$ and H_2O_2 production at the level of Nox enzymes [8,10]. Examples include epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), which mediate ROS-dependent pro-proliferative MAP-kinase signalling *via* Nox enzyme activation [90]. These stimuli can also lead to mitochondrial ROS production, which can be affected by oncogene expression and changes in metabolic flux and oxygen tension of the tumor microenvironment [91]. These changes are able to elicit ROS production within tumor cells and tumor associated cells such as fibroblast and immune cells. However, it remains difficult to quantify changes in oxidant levels within the tumor microenvironment *in vivo*. During redox signaling, the changes in ROS/RNS generation and subsequent redox-signaling seem to occur in a spatiotemporal context and appear to be dynamically regulated. Much like other second messengers, it is likely that the type of oxidant species, amount and location are of importance in determining the eventual cellular response elicited [13,15,21,22,92]. The existence of cellular ROS scavengers and antioxidants means that reactions are largely reversible, a necessary feature of cellular signaling (Figs. 2 and 3). While beyond the scope of the present review, it is becoming evident that the levels of certain antioxidants, such as Sod2 and GSH are increased in tumor cells, and potentially necessary to ensure survival during metastatic progression, either to cope with excess redox stress or to contribute to alterations in the redox status and redox signaling of cancer cells [93–100].

While several amino acid residues have a higher propensity to react with oxidants (cysteine, selenocysteine, methionine, tyrosine, tryptophan, histidine), one of the most important redox signaling targets and most widely studied are the thiolate anions of cysteine residues (Fig. 3). Given their pKa, cysteine residues exist as both the sulfhydryl and thiolate at neutral pH. The thiolate is rapidly oxidized to sulfenic acid in the presence of oxidizing agents. From this, a number of modifications are commonly formed, including the reduction and disulfide bond formation with other thiols, either intra- or inter molecularly [14,21]. Reaction with $^{\bullet}NO$ to form S-nitrosothiols can also be an important modification that alters protein function. Cysteine residues are central to catalytic function and structural properties of proteins, as they act as nucleophiles in chemical reactions and facilitate disulfide bond formation during protein folding. Hence, cysteine redox modifications and the ability to "reverse" these reactions highlights their importance as cellular signaling intermediates. The degree and reversal of oxidative thiol modifications is often dependent on cellular factors including pH and the levels of enzymes like glutaredoxin (Grx) and thioredoxin (Trx) which reduce disulfides back to the cysteine thiol [14].

Due to its ability to diffuse within the cell, traverse biological membranes and a relatively longer half-life than other ROS, it has been suggested that H_2O_2 may be a suitable second messenger and major contributor to redox signaling within cells [13]. While H_2O_2 has been implicated as a ROS second messenger in a manner similar to Ca^{2+}, the role of individual oxidant and reactive nitrogen species as *bona fide* signaling molecules or second messengers is still under investigation. Many reactive oxygen species, such as $O_2^{\bullet-}$ are short lived and therefore either directly oxidize targets within close proximity to their site of production (e.g. near Nox of the plasma membrane or ETC complexes near mitochondrial membranes), or rapidly react to form secondary species, such as $ONOO^-$, or are enzymatically/spontaneously dismuted to H_2O_2. Interestingly, based on reaction kinetics, it is thought that H_2O_2 may not be able to directly oxidize cysteine residues of many identified target proteins, such as phosphatases. Instead it has been proposed that reaction with Prx likely quickly consumes most of the H_2O_2 produced within the cell. This would limit free H_2O_2 diffusion and direct oxidation of proteins, such as phosphatases, that are potential targets of H_2O_2 redox signaling (Fig. 2). This conclusion is based on the rate constant of the reaction between H_2O_2 and Prx, and the relatively high abundance of Prx enzymes within cells and compartments such as mitochondria [22,101]. Instead of direct oxidation of signaling proteins by H_2O_2, Prx may act as an intermediate or "redox relay" to carry out subsequent redox-mediated signaling, including disulfide exchange [22,101]. Alternatively, if H_2O_2 production is very high in specific nano-domains of the cell, irreversible hyperoxidation or other post-translational modifications inactivating the local Prx pool could lead to localized H_2O_2 build-up that could directly oxidize thiols of target proteins within its vicinity [102].

Redox regulation of Ca^{2+} homeostasis has been demonstrated in a variety of contexts and shown to occur *via* direct oxidation of Ca^{2+} channels and channel regulators, as described in more detail below. While not discussed in depth, indirect modulation of Ca^{2+} signaling may also occur through the action of redox-mediated activation of either gene transcription or cell signaling pathways, such as oxidation and subsequent inhibition of protein tyrosine phosphatases or activation of kinases [14,15]. An example of this is the redox regulation of the phosphatases PP2B and PP2A, which are responsible for the dephosphorylation and regulation of the Ca^{2+}/Calmodulin-dependent kinase II (CaMKII). Oxidation leads to PP2A/B inactivation and a subsequent increase in CaMKII phosphorylation, thereby influencing downstream Ca^{2+} signaling pathways [103]. In contrast kinases are often activated by oxidation. In this context, oxidation and activation of PKC and PKA can further enhance phosphorylation of CaMKII resulting in an overall net effect of redox stimulated CaMKII phosphorylation and activation in response to increases in ROS [104].

Ca^{2+} channels can be directly affected by oxidation to alter Ca^{2+} signaling. Cysteine residues within Ca^{2+} channels and activators of Ca^{2+} channels such as the Stromal Interacting Molecule-1 (STIM1) are susceptible to oxidation and protein modifications, including glutathionylation and di-sulfide bond formation (Fig. 3), which can affect protein conformation and activity. STIM1 is an important Ca^{2+} sensor located in the ER, which activates ORAI store-operated Ca^{2+} entry (SOCE) channels. Interestingly, it appears that different redox modifications of STIM1 can elicit divergent consequences on protein function. Although further studies are required to verify these observations, it has been shown that glutathionylation of STIM1 cysteine residues results in store-independent activation of ORAI by STIM1, while STIM1 disulfide formation may decrease Ca^{2+} entry [105,106]. Similarly, TRPC5 channel oxidation has been shown to yield different redox modifications and consequential channel function, including S-nitrosothiol formation, glutathionylation and inter- and intra-molecular disulfide bond formation [107–110].

A caveat of many earlier studies examining the role of oxidation on channel function is the lack of attention to the amounts of exogenous ROS applied, and whether these reflect physiologically or pathophysiologically relevant levels. For example, exogenous application of H_2O_2 in the low mM range is often used to demonstrate the role of oxidants in activating Ca^{2+} influx, which is a dose that elicits cell death within a matter of hours in most cells, and likely represents redox stress. As it is difficult to mimic the spatio-temporal sub-lethal increases commonly associated with redox-signaling hubs, experimentally applied bolus doses may hence lead to very different oxidation events and cellular outcomes. An example of this is irreversible sulfonic acid formation, which may inactivate proteins indefinitely, while intra- or inter-molecular disulfide formation can be reversed by protein disulfide reductases such as thioredoxins (Fig. 3). In addition, the role of each specific reactive species is largely lacking. Reactive oxygen and nitrogen species differ widely in their ability to oxidize proteins and react with other cellular component including metals such as iron. Pro-

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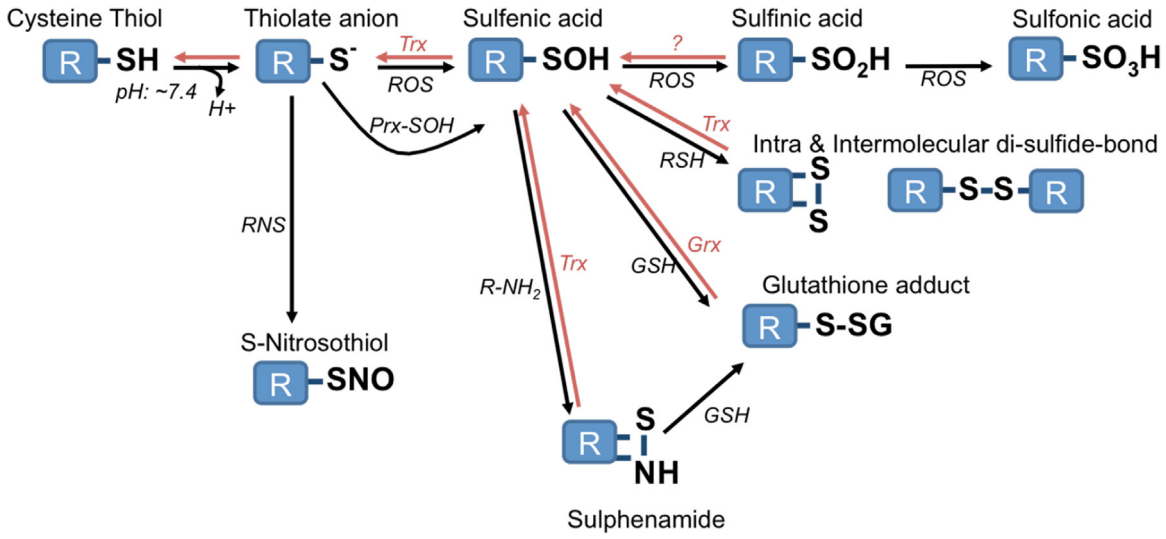

Fig. 3. Oxidative modification of cysteine thiols. The reversible nature of most modifications by the Thioredoxin (Trx) and Glutaredoxin (Grx) system highlight their role in cellular signaling. Rather than direct oxidation of cysteine residues by ROS, it is thought that intermediate redox sensors, such as Peroxiredoxins (Prx), may be the first target of ROS and subsequently carry out redox modifications of target proteins.

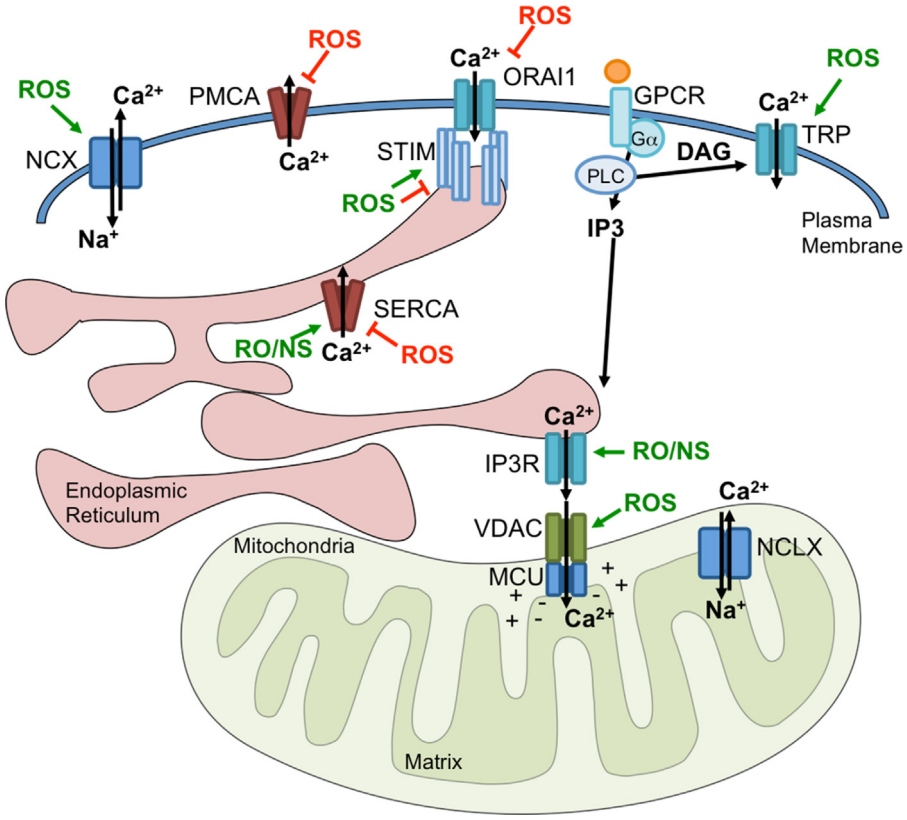

Fig. 4. Reactive oxygen and nitrogen species can directly influence the activity of Ca^{2+} regulators at multiple locations within the cell. Studies have demonstrated either direct or indirect redox regulation of ion channels and pumps at the plasma membrane, ER and Mitochondria (activation indicated in green; inhibition indicated in red). Plasma Membrane channels and pumps represented include Transient Receptor Potential (TRP) Channels, ORAI, Plasma Membrane Ca^{2+} ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX). The ER Ca^{2+} sensor and ORAI regulator, STIM1 has also been demonstrated to be under redox control. Similarly, ER-localized Inositol 1,4,5-trisphosphate (IP_3) receptors (IP3R) and the Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), as well as mitochondria-localized Voltage-dependent anion channel (VDAC) and the Mitochondrial Ca^{2+} Uniporter (MCU) activity are influenced by ROS. While much of this information has been gleaned in other model systems, the role of these redox modifications in the context of cancer require further investigation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tein modifications may hence differ based on the cellular redox environment and these differences are observed in studies examining the redox regulation of ion channels, as illustrated below. Although many pathways of Ca^{2+} regulation and redox signaling have been separately described in cancer cells, their interplay has not been investigated in great detail in the context of this disease. Below we give examples of redox regulation of Ca^{2+} modulators, as they were discovered in various disease models, and speculate on potential consequences of this interplay in cancer. Examples of studies demonstrating direct cysteine redox modifications of Ca^{2+}

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channels and channel regulators, as well as indirect mechanisms of redox regulation are described, as they pertain to Ca^{2+} regulators of the plasma membrane, ER and mitochondria (Fig. 4). From these studies, one can deduct that there are likely two overarching themes that emerge for the redox-Ca^{2+} interplay in cancer cells: 1. Coordinated ROS and Ca^{2+} signals are required for apoptosis initiation at the mitochondrial ER interface, with emerging evidence suggesting that this interplay is altered in cancer cells to enhance apoptosis resistance. 2. The interplay between Ca^{2+} and ROS influences cellular signaling cascades that promote proliferation and metastasis. The latter scenario likely involves coordinated changes in localized, sub-lethal concentrations in both ROS and Ca^{2+} levels.

3. Redox regulation of cellular Ca^{2+} homeostasis at the plasma membrane

Regulation of Ca^{2+} homeostasis at the cell membrane occurs through a number of channels and pumps that can be activated by a variety of signals including mechanical and chemical stimulants, ligand-dependent receptor activation and subsequent generation of second messengers, intracellular Ca^{2+} store depletion, and oxidants. It is clear from the literature that the cellular context and differences in the spatio-temporal nature of the oxidant signal influences the activity of plasma membrane channels and subsequent Ca^{2+} dependent cellular responses. For example, high levels of ROS or "oxidative stress" can inhibit cytoplasmic Ca^{2+} extrusion [111,112] and enhance Ca^{2+} entry through TRP channels located at the plasma membrane [113–118] (Fig. 4). This enhances intracellular Ca^{2+} levels, which are thought to contribute to the induction of cell death. In contrast, it is conceivable that sublethal and localized ROS production, might initiate pro-tumorigenic Ca^{2+} signaling *via* activation of cell surface Ca^{2+} channels, such as proliferation [119], and wound healing [86]. In both oxidative stress and redox signaling, the location of cell surface Ca^{2+} channels clearly makes these an excellent target for sensing redox changes in the intra and extracellular environment [120].

Many of the stimulatory cues that activate plasma membrane channels are often altered in the tumor microenvironment and it is therefore conceivable that altered activity of plasma membrane associated Ca^{2+} channels and pumps is a common phenotype of cancer cells [120]. Moreover, remodeling of ion channel expression and alterations in Ca^{2+} signaling by plasma membrane cation channels, including TRPC1, TRPC3, TRPC6, TRPM2, TRPM7, TRPV6, ORAI1 and ORAI3 have been implicated in enhanced proliferation of tumor cells [16,121]. Stimuli that activate these channels in cancer cells range from cholesterol (TRPM7) and GPCR agonists (ORAI1 and TRPC channel isoforms) to constitutive activation (TRPV6). In turn, this leads to activation of Ca^{2+}-dependent signaling including the calcineurin/NFAT, CaM Kinase and Akt/Erk pathways to increase cell cycle progression (for reviews see [16–19]). As seen from the examples highlighted below, a common theme in cancer appears to be that tumor cells utilize redox regulated Ca^{2+} signals to drive proliferation and invasion, while they avoid sustained Ca^{2+} fluxes associated with apoptosis, which are generally elicited by oxidative stress.

3.1. Transient receptor potential (TRP) cation channels

Trp refers to a large gene family encoding transient receptor potential (TRP) proteins, which form mostly plasma membrane non-selective cation channels. However, some TRP channels function as calcium release channels in internal organelles [122]. TRP channels have variable activation and gating mechanisms and play a crucial role in a large number of cellular and physiological functions, ranging from sensory signaling to signaling pathways that control contraction, growth, migration and cognition. In mammals, the TRP superfamily consists of 28 *TRP* genes that can be divided into 6 families based on sequence homology: Canonical TRP (TRPC), Vanilloid TRP (TRPV), Melastatin (TRPM), Ankyrin TRP (TRPA), Mucolipin TRP (TRPML) and Polycystin TRP (TRPP). There are only 27 TRP genes in humans, with TRPC2 (involved downstream pheromone receptor signaling in rodents) being a pseudogene in humans [123]. A number of TRP channels have been shown to alter their activity in response to oxidative stress, either indirectly (e.g. TRPM2) or by direct channel oxidation (e.g. TRPV1, TRPC1, TRPM7) [124,125]. Studies have demonstrated TRP channel involvement in cell proliferation, migration, angiogenesis, chemokine production and autophagy [17,18,120,126,127] and there is some evidence that redox regulation of TRP channels may play a role in cancer.

3.1.1. Redox regulation of TRPM2 in cancer

The melastatin TRP subfamily member, TRPM2 (formerly named TRPC7/LTRPC-2) has been a particular focus due to its regulation by oxidants. In other pathologies besides cancer TRPM2 has been linked to mediating Ca^{2+} influx during apoptosis, including endothelial and neuronal cell death, and male-specific ischemic injury in response to oxidative stress [113,116,128,129]. TRPM2 can also directly influence ROS production at the level of Nox enzymes. It has been shown that TRPM2 regulates Rac1 and Nox activation to mediate ROS production during ischemic kidney injury [130]. TRPM2 was originally identified as a potential tumor suppressor [131]. As such, in an attempt to take advantage of the pro-apoptotic role of TRPM2, forced expression of the channel was shown to enhance cell death in A172 human glioblastoma cells [132]. However, as detailed below, the role of TRPM2 appears to be complex and multifaceted in cancer (Fig. 5).

Unlike other TRP family members, the non-selective cation channel TRPM2 is not directly redox modified, as demonstrated by a lack of methionine or cysteine oxidation [116]. A number of studies have shown that H_2O_2 activates TRPM2 channel activity in an indirect manner. Treatment with a range of H_2O_2 doses (100 μM–3 mM) leads to ADP-ribose generation, a known activator of TRPM2, which directly binds its C-terminal domain to cause activation [114,133,134]. ADP-ribose is formed in both the mitochondria and in response to cellular stress/DNA damage by poly(ADP-ribose) polymerases (PARP) in a nicotinamide adenine dinucleotide (NAD)-depended manner. Both ADP-ribose generating pathways have been implicated in eliciting indirect H_2O_2 regulation of TRPM2 [117,133,134]. Interestingly, TRPM2-mediated Ca^{2+} influx during apoptosis elicits caspase activation and PARP cleavage. This suggests a potential negative feedback mechanism, where decreases in PARP-mediated ADP-ribose generation could dampen TRPM2 activity [135]. In addition to TRPM2 activation by ADP-ribose, it was shown that PKCα-mediated phosphorylation of a short TRPM2 isoform (TRPM2-S) is H_2O_2 dependent and leads to Ca^{2+} influx in endothelial cells [136]. The authors suggest this to be an alternate mechanism for redox regulation of TRPM2 during the initiation of apoptosis, as TRPM2-S phosphorylation initiates dissociation of TRPM2-S from the full length form TRPM2-L (long isoform). This confirms the potential role of TRPM2-S as a dominant negative regulator [115]. TRPM2-S lacks 4 transmembrane domains, and was previously demonstrated to inhibit ADP-ribose dependent channel activation by binding TRPM2-L [115]. In apparent contrast to Hecquet et al., the investigators noted that TRPM2-S has the ability to blunt H_2O_2 mediated activation of TRPM2-L at the cell membrane. Here enhanced expression of TRPM2-S inhibited H_2O_2 mediated apoptosis in cells expressing TRPM2-L, by blunting ADP-ribose binding that is induced in response to H_2O_2 [115].

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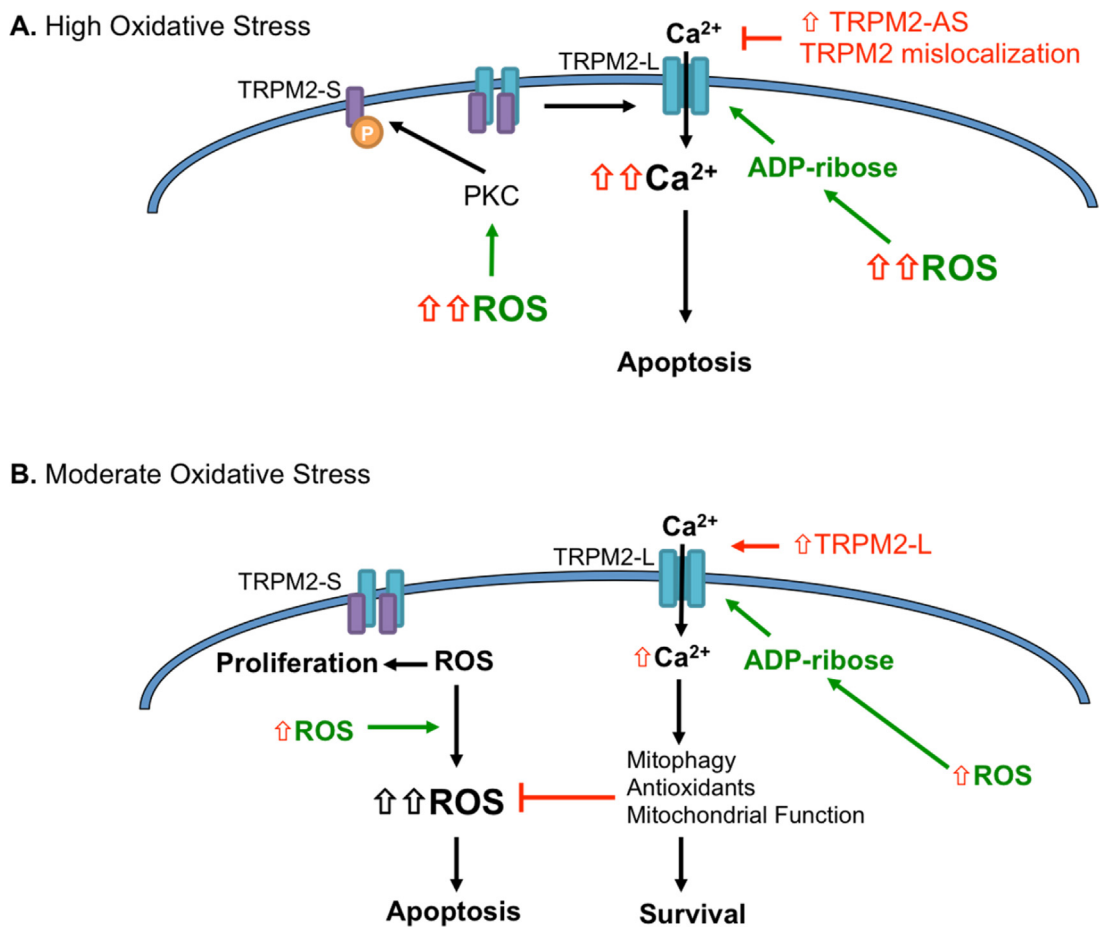

Fig. 5. Divergent roles of TRPM2 under redox regulation **A.** Under high oxidative stress TRPM2 is activated to induce apoptosis. Several mechanisms for this have been proposed, including ROS-dependent increases in ADP-ribose production. Alternatively, ROS-dependent PKC phosphorylation of the TRPM2-S isoform, leads to dissociation of this potential dominant negative splice variant from TRPM2-L to induce TRPM2-L channel activation. Cancer cells have developed several mechanisms to avoid TRPM2-L channel activation in response to high oxidative stress, including TRPM2 mislocalization to the nucleus and high expression of a TRPM2-antisense (AS) mRNA. **B.** Under moderate oxidative stress, which may be observed in response to pro-oxidant changes, such as nutrient deprivation and hypoxia in the tumor microenvironment, TRPM2-L has a pro-survival function. Cancer cells expressing higher levels of the TRPM2-S isoform were shown to operate under a higher cellular ROS status, which drives pro-proliferative signaling. However, when challenged with moderate ROS stress TRPM2-S cells are unable to elicit necessary Ca^{2+}-dependent pro-survival pathways, unlike TRPM2-L expressing cells. These studies show that expression of TRPM2-L presents cells with a survival advantage under moderate oxidative stress, and highlights the importance of TRPM2 isoform expression and the levels of ROS stress in determining cancer cell fate.

Several lines of evidence suggest that tumor cells are either able to prevent ROS mediated TRPM2 activation during apoptosis initiation, or to use redox regulated TRPM2-dependent Ca^{2+} signaling to aid in tumor growth and resistance to therapy (Fig. 5). An example of the former is highlighted by the interesting observation that expression of a non-coding antisense TRPM2 RNA (TRPM2-AS) is increased in prostate cancer. This appears to protect cells from TRPM2-mediated apoptosis and cellular redox-stress [137,138]. High expression of TRPM2-AS was also associated with poor patient outcome and increased proliferation, while decreasing expression of TRPM2-AS initiated apoptosis and inhibited tumor growth of prostate cancer cells *in vivo* [137]. Although the investigators did not directly measure if this resulted in a change in TRPM2 currents, mRNA levels of TRPM2 were increased in response to TRPM2-AS knockdown [137]. In apparent contrast with this work, high TRPM2 coding mRNA levels were shown to correlate with increased proliferation in another prostate cancer study [139]. However, upon further exploration the investigators noted substantial mislocalization of TRMP2 to the nucleus. This may represent an alternate mechanism by which cancer cells evade pro-apoptotic TRPM2 channel activation at the cell surface. The authors suggest that this enhancesd tumor cell proliferation, although the mechanisms for the latter were not clearly delineated [139].

Given the pro-apoptotic function of TRPM2 it appears counterintuitive that high TRPM2 expression has been observed in a number of cancer types, including neuroblastoma, prostate cancer and melanoma [138–141]. The studies described below suggest that TRPM2 elicits a protective role in response to sublethal increases in oxidative stress, which is dependent on cellular context, amounts of reactive oxygen species, and levels of TRPM2 isoform expression, and highlights the context-dependent nature of the ROS/Ca^{2+} interplay [142]. Expression of the above mentioned TRPM2 splice variants may influence the Ca^{2+}/ROS interplay mediated by TRPM2 in cancer cells and fine tune signaling to proceed to either a pro-proliferative or pro-apoptotic path (Fig. 5). Miller and co-workers showed that expression of both the short TRPM2-S and long TRPM2-L variants are increased in neuroblastoma cells, and that these two isoforms have divergent roles in cancer and in response to variable levels of oxidative stress [140,141]. TRPM2-S cells were shown to have higher basal levels of cellular ROS than TRPM2-L cells. In turn, this was associated with oxidation and inactivation of the phosphatase and tensin homolog (PTEN) and consequential increases in the PI3K/Akt and Erk pathways, leading to higher proliferative rates in TRPM2-S cells [141]. Interestingly, TRPM2-S cells were unable to cope with the addition of exogenous oxidative stress, which may again point to the fact that an increased

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in intracellular steady state ROS is deleterious to cancer cells once faced with additional exogenous ROS. In contrast, TRPM2-L expressing cells were protected against exposure to 50–100 μM H_2O_2. Mechanistically, this was shown to be due to TRPM2-L-dependent expression of the transcription factor FOXO3a, and consequential increases in the FOXO3a-regulated mitochondrial antioxidant enzyme Sod2 [141]. Moreover, TRPM2-L expressing cells also expressed high levels of the glucose transporter GLUT-1, a phenotype of glycolytic tumor cells. In a subsequent paper the authors showed that neuroblastoma cells with high TRPM2-L expression were more tumorigenic than cells expressing TRPM2-S using *in vivo* xenografts model [140]. TRPM2-L expressing cells had increased HIF-1/2α expression and this also conferred chemoresistance to doxorubicin [140]. In essence, these studies suggest that the expression of TRPM2-S may be beneficial to rapidly proliferating cells under optimal conditions, including adequate oxygen and nutrients supply, while under stress conditions the expression of TRPM2-L is essential in initiating pro-survival pathways such as HIF and antioxidant enzyme expression. This was further demonstrated in TRPM2-L CRISPR/Cas9 deleted cells, which were less tumorigenic and more susceptible to doxorubicin cytotoxicity [143]. The pro-tumorigenic action of TRPM-L was again shown to be due to its role in maintaining HIF stabilization, and by maintaining mitochondrial redox balance through expression of FOXO3 and Sod2. This preserved mitochondrial function and ATP production [143]. The complex, yet intriguing, relationship between the TRPM2 isoforms requires further investigation to ascertain if the ratio of TRPM2-L to TRPM2-S is altered in cancers and if this is associated with patient outcome and chemoresistance.

Redox-regulation of TRPM2 may also be important during immune response and inflammation, and shown to be necessary for NLRP3 inflammasome activation, monocyte chemokine production for the recruitment of neutrophils, and bacterial clearance [144–146]. In contrast, it has been shown that activation of TRPM2 which is non-selective and conducts a substantial amount of Na^+ ions (in addition to Ca^{2+}) can lead to plasma membrane depolarization in phagocytes, which results in the suppression of Nox-dependent ROS production in response to endotoxin to suppress the inflammatory process [147]. This negative feedback mechanism may ultimately protect cells and tissues against sustained redox stress during inflammation. Melendez and co-workers described an interesting mechanism whereby the Gram-negative bacterium *Francisella tularensis* utilizes the antioxidant ability of its catalase to limit TRPM2-mediated Ca^{2+} entry in host macrophages, thus inhibiting actin reorganization and cytokine production by these cells [148]. The role of the TRPM2-redox/Ca^{2+} axis during tumor clearance by immune cells would be of interest to investigate in future studies.

3.1.2. TRP – nox interplay

Unlike the indirect regulation of TRPM2 by ROS, other members of the TRP ion channel family have been shown to be directly redox modified [124]. The canonical TRP (TRPC) channel family is primarily regulated by Phospholipase C (PLC) coupled receptors and the downstream actions of Phosphatidyinositol-4,5 bisphosphate (PIP_2) hydrolysis, Diacylglycerol (DAG) production and rise in cytosolic Ca^{2+} [150–152]. ROS/RNS have been shown to regulate a number of TRPC family members, including TRPC3, 4 and 5, which are activated following oxidative stress in a number of cell lines [153–155]. While there are studies to show the importance of TRPC channel activity in regulating cancer cell migration, proliferation, epithelial-to-mesenchymal transition, angiogenesis and chemoresistance [109,156–161], studies have not focused on the redox regulation of these in the context of an enhanced ROS tumor cell milieu or in response to ROS-generating conditions emanating from the tumor microenvironment. This is clearly an area that requires further attention.

A few divergent examples highlight additional roles for TRP-redox regulation in relation to their interaction with Nox. Again, the resultant cellular consequences are cell type specific. For example, increased TRPC6 activation by insulin initiates kidney podocyte apoptosis in a Nox4-derived H_2O_2-dependent manner [38,149], while B-cell lymphoma cell proliferation is dependent on TRPC6 channel activation by Nox2 generation of $O_2^{\bullet-}$ [119] (Fig. 6). In these cancer cells cholesterol increased expression of the Nox2 subunitsp47-phox and gp91-phox, this led to a concomitant increase in expression and activation of TRPC6, and intracellular Ca^{2+} increases. This was shown to be dependent on Nox2 mediated ROS production and was abrogated by the cholesterol lowering drug, lovastatin [119]. The interplay between TRP and Nox proteins may also be important for eliciting unwanted side effects of chemotherapeutics, including ototoxicity, which results in damage to the inner ear and hearing loss. For example, the interplay between Nox3 and TRPV1 appears to be involved in initiating apoptosis of outer hair cells of the cochlea in response to Cisplatin treatment [162,163]. These examples highlight that redox regulation of TRP channel activity can have very different consequences, depending on the cellular context, specific Nox isoform interaction and type of ROS involved.

3.1.3. TRP channels and regulation of autophagy

Recently, attention has been placed on the mucolipin TRPML channel family members in the regulation of autophagy, given their localization to lysosomal membranes and influence on Ca^{2+} signaling in these organelles [3,164]. Autophagy is regulated by a number of cellular stressors, including nutrient deprivation, and the unfolded protein response/ER stress, which are closely associated with oxidative stress [44]. TRPML channels are localized to the lysosomal membrane and mediate Fe^{2+} and Ca^{2+} release from these organelles. TRPML channels are important for lysosomal pH balance, endo-lysosome formation and trafficking, and can respond to activation by $PI(3,5)P_2$ and changes in pH [165,166]. TRPML1 was recently shown to be directly activated by H_2O_2 to elicit lysosomal Ca^{2+} release. This was shown to increase generation of autophagosomes, as visualized by enhanced accumulation of the autophagy markers Microtubule-associated proteins 1A/1 B light chain 3B (LC3-II) and Lysosomal-associated membrane protein 1 (Lamp1). Mechanistically, TRPML1 mediated calcineurin-dependent dephosphorylation of the transcriptional regulator of lysosomal and phagosomal biogenesis, Transcription Factor EB (TFEB), which is usually maintained in an inactive from by mTOR-mediated phosphorylation [167–169]. Interestingly, this regulation was specifically related to redox regulation of autophagy and proposed to represent one mechanism for cells to remove damaged mitochondria in response to oxidative stress [168]. Although TRPML1 can also respond and be upregulated in response to nutrient stress [169], the consequences of this regulation in cancer cells has not been investigated. It is possible that TRPML1 could trigger autophagy more readily in cells with elevated redox thresholds and may be advantageous to cancer cells to enhance survival in situations of nutrient and redox stress. Targeting TRPML1 could present a novel therapeutic strategy, as cutting off various nutrient supplies to tumors is increasingly being explored as a novel mechanism to kill cancer cells.

Non-selective Ca^{2+}-permeable TRP channel isoforms at the plasma membrane are also involved in eliciting Ca^{2+} signaling that controls autophagy. It was shown that pro-survival induction of autophagy in thymocytes in response to capsaicin was TRPV1-dependent and requires both intracellular Ca^{2+} rise and ROS generation, which was necessary for 5′ adenosine monophosphate-activated protein kinase (AMPK) activation,

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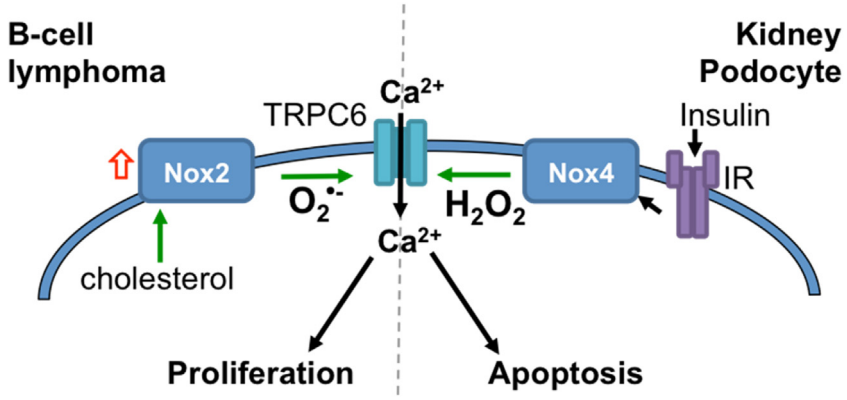

Fig. 6. Divergent cellular consequences of Nox interactions with TRPC6 highlights the context dependence in ROS-Ca^{2+} signaling. Following insulin receptor engagement Nox4 activation leads to H_2O_2 production in podocytes, which induces TRPC6 activation and Ca^{2+} influx that drives apoptosis [38,149]. Conversely, Nox2 expression in response to cholesterol induces redox-dependent expression and activation of TRPC6, required for proliferation of B-cell lymphomas [119].

Autophagy Related 4C Cysteine Peptidase (Atg4C) expression, and induction of Atg6/Beclin-1-dependent autophagy [170]. Similarly, TRPC1 dependent Ca^{2+} entry was shown to initiate autophagy in response to hypoxia and nutrient deprivation [171]. A recent report suggests that TRPM2 activation results in Beclin1 phosphorylation *via* CaMKII to decrease autophagy, making hepatocytes more susceptible to cell death in response to oxidative stress [172]. This contrasts with the role of TRPM2-L mediated induction of HIF-1α discussed above, which was shown to increase autophagy in a BNIP3 dependent manner to enhance survival of neuroblastoma cells [140]. Whether changes in TRP channel expression in cancer cells also alter their ability to initiate autophagy in response to redox activation requires further exploration.

3.2. Store-operated calcium entry (SOCE)

Store operated Calcium entry (SOCE) is a major mechanism for Ca^{2+} regulation in non-excitable cells, and is increasingly implicated in mediating Ca^{2+} signals that control a large number of normal physiological processes and its dysfunction contributes to several diseases [173,174]. SOCE is activated following Ca^{2+} depletion from ER stores, through phopshtaidylinositol-1,4,5-trisphosphate (IP_3) Receptor Ca^{2+} release channels or Ryanodine receptor (RyR) in the case of muscle. IP_3 is produced by activation of plasma membrane receptors to hormones, growth factors and neurotransmitters that couple to phospholipase C (PLC) isoforms leading to hydrolysis of phosphatidylinositiol-4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol (DAG). Store depletion activates the store-operated current called Ca^{2+} release activated Ca^{2+} (CRAC). CRAC currents are mediated by the Orai protein family (ORAI1, 2 & 3), and their activation is regulated by STIM1 and STIM2 proteins, which are ER-localized Ca^{2+} sensors [174–177]. In addition to the ER, mitochondria are involved in the regulation of SOCE, either due to their Ca^{2+} buffering capability [178,179] or through mitochondria-derived redox-signaling [180]. The plasma membrane, mitochondria and ER are associated with ROS production, and hence it is not surprising that redox regulation of ORAI channels and STIM proteins could be of importance in a variety of cell types. In normal physiology, the SOCE pathway mediates Ca^{2+} signaling important for maintenance of cell function in non-excitable as well as excitable cells. In cancer, STIM and ORAI isoforms display increased expression in a number of tumor types and have been associated with signaling pathways that positively regulate cancer cell proliferation, migration, invasion, and chemoresistance [181–189]. Further, a number of studies have demonstrated the importance of the SOCE pathway and its regulators in endothelial progenitor cells, VEGF-mediated endothelial tube formation and endothelial cell proliferation [190–192], and this may similarly be important for tumor angiogenesis [193,194]. Hence, it appears that enhanced SOCE generally supports pro-tumorigenic and pro-metastatic phenotypes.

The interplay between SOCE and ROS is multifaceted and the role of this regulation in cancer is only starting to emerge. However, from other cell model systems it is evident that redox-dependent modifications of ORAI and STIM play a role in the regulation of SOCE (for detailed review see [195]). Again, consequences of the ROS-SOCE Ca^{2+} interplay depend on the levels of ROS that cells are exposed to. In circumstances of oxidative stress, SOCE leads to the initiation of apoptosis. For example, neuronal cell death in response to ischemia is dependent on STIM2-mediated Ca^{2+} influx [196], and vascular dysfunction in an acute lung injury model in response to LPA is dependent on Nox2-derived ROS and subsequent redox-mediated activation of STIM1-depedendent SOCE [197]. As illustrated below, oxidation of STIM and ORAI can elicit both positive and negative effects on SOCE. Clearly, the source of ROS, levels and duration of this redox signal will determine the eventual impact on SOCE. For example, domains of localized ROS production at the ER and mitochondria may exclusively affect SOCE *via* redox activation of STIM1 and hence lead to pro-tumorigenic Ca^{2+} signaling, while exogenous redox stress sensed by plasma membrane localized ORAI channels may block SOCE and attenuate proliferation of tumor cells and also make tumor cells more susceptible to redox stress (Fig. 7) [198]. It is also possible that ROS mediate STIM translocation and SOCE activation indirectly, through redox activation of ER Ca^{2+} regulators IP_3R and SERCA, which are also redox sensitive (See Section 4). While detailed studies are required to unravel the complex nature of redox regulation of STIM and ORAI in the context of cancer cells and their tumor microenvironment, the following examples shed light on the potential role of the SOCE-ROS interlay in this context.

3.2.1. Redox regulation of STIM1

STIM1 has two cysteines (C49, C56) that have been demonstrated to be redox sensitive (Fig. 7**A**) [105,106]. Redox modification of cysteine 56 was shown to lead to glutathionylation, which resulted in decreased STIM1 Ca^{2+} binding, thereby mediating STIM1 oligomerization and activation of SOCE, independent of Ca^{2+} ER store depletion [105]. In somewhat contradictory work, Prins et al. demonstrated that oxidation of cysteine 56 results in intramolecular disulfide bond formation with cysteine 49. These two cysteines were also shown to be necessary for binding ERp57, an ER localized oxidoreductase [106]. ERp57-STIM1 interaction leads to decreased activation of STIM1 oligomerization and a decrease in SOCE. Further work is required to demonstrate that disulfide bond formation

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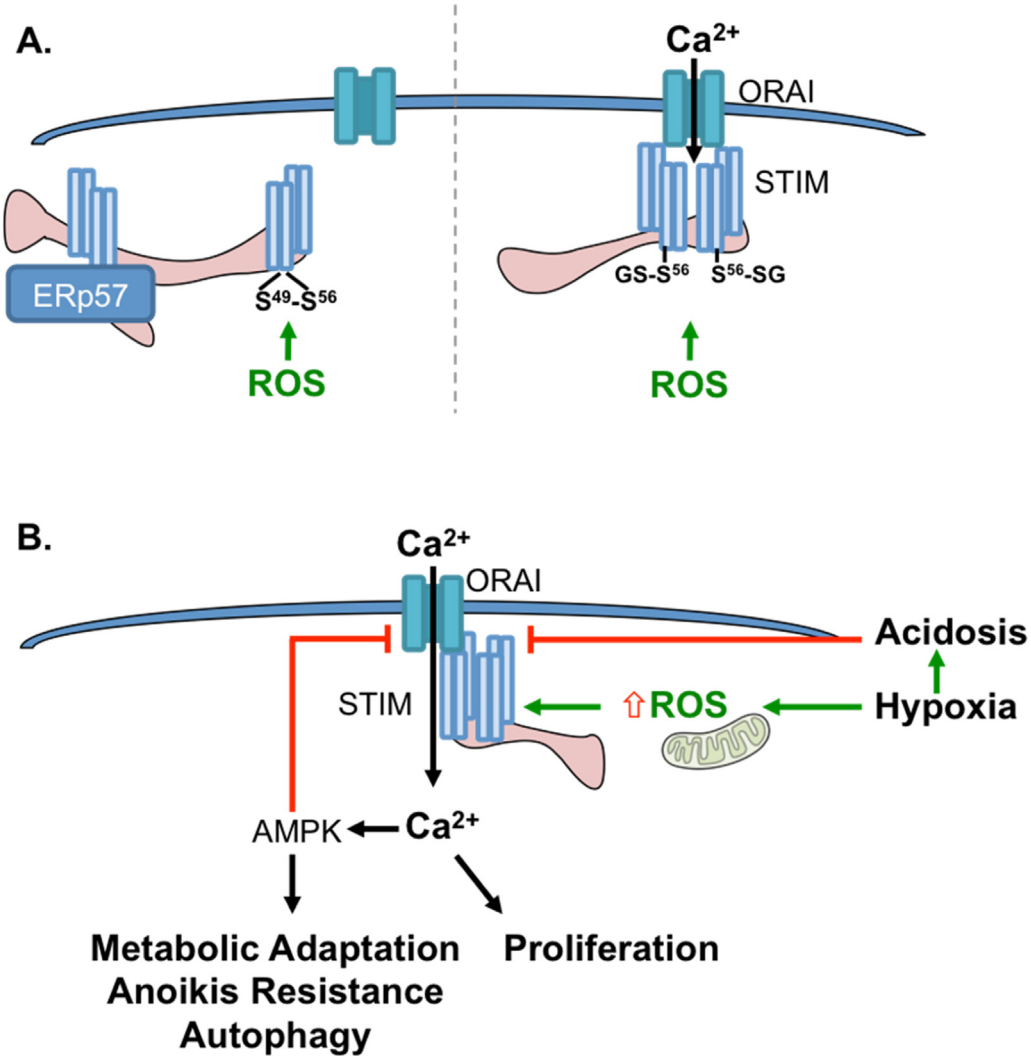

Fig. 7. A. Redox Modifications of STIM1. **A.** STIM1 has the potential to form intra-molecular disulfide bonds (left) at Cysteine 49 and 56 following oxidation, which are also important for ERp57 binding. Both mechanisms are thought to inhibit STIM oligomerization and SOCE [106]. Conversely, glutahionylation (right) of the same cysteine residues is thought to decrease Ca^{2+} STIM binding to mimic store depletion and facilitate Stim oligometization and Orai interaction to initiate SOCE [105]. enhance inhibition reand glutathione adducts, which may result in either SOCE inhibition or activation. **B.** Potential consequences of STIM1 redox regulation in tumor cells in response to Hypoxia. Acute hypoxia induces mitochondrial ROS production, resulting in STIM1 puncta formation and SOCE activation. The resultant Ca^{2+} signal is important for tumor cell proliferation [199], and activates AMPK, a known regulator if metabolic adaptation and autophagy [200,201]. Prolonged AMPK activation and the development of acidosis in response to long-term hypoxia may represent negative feedback mechanisms to shut-down SOCE [202,203].

is similarly sufficient to block STIM1 puncta formation and activation of ORAI1. Moreover, it is of interest to investigate if the local ER redox status and glutathione pool resembles a potential redox switch for the suppression or activation of SOCE, respectively. These differences in STIM1 cysteine redox modifications may be cell type specific, but highlight that disparities in redox modifications at the same ER-localized cysteine could result in different cellular outputs related to STIM1-regulated SOCE. Redox regulation of STIM1 cysteine residues outside the ER luminal domain have not been investigated, however mutation studies suggest that these could also affect SOCE [195]. Different redox environments and pH between the ER lumen and the cytosol could also influence the difference between oxidation of luminal and cytosolic cysteine residues. Similarly, cysteine residues residing on either the inside or outside of plamsa membrane localized channels may be differentially affected by differences in intracellular and extracellular ROS levels, respectively, which may be influenced by differential antioxidant enzyme expression in subcellular compartments and ROS scavengers such as ascorbate in the extracellular space.

There is also evidence to suggest that STIM2, which is more sensitive to smaller drops in ER Ca^{2+} [204], may be oxidized at C725 in vitro, and may be important for SOCE regulation in hypoxia [195,205]. Recent work is starting to unravel the role of STIM2 in regulating SOCE [206–210], and differential expression of STIM2 relative to STIM1 may be a phenotype of certain tumors, where higher STIM1:STIM2 ratio may suggest a worse prognosis in some cancer types [183,211–213]. However, further studies are needed to illustrate the mechanistic consequences of STIM2 expression and potential redox regulation in cancer.

CRAC channel activity appears to be sensitive to stress signals that are associated with changes in cellular ROS and are a common phenotype of adaptations observed in cancer cells, including hypoxia and nutrient stress. Redox modification of SOCE has been implicated in eliciting cellular outcomes in response to hypoxia (Fig. 7**B**). Hypoxia increases mitochondrial ROS production at complex III of the electron transport chain (ETC), and this was shown to cause translocation of STIM1 to the plasma membrane and CRAC channel opening [200,201]. Hypoxia may also directly regulate STIM1 expression. A recent report demonstrated that STIM1

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expression is positively regulated by HIF-1α in response to hypoxia in hepatocarcinoma cells, and that the resultant increase in SOCE is necessary for hypoxic tumor growth [199]. While hypoxia appears to influence the redox activation and expression of STIM1, hypoxia is also able to blunt SOCE *via* disruption of STIM1-ORAI1 interaction and ORAI1 pore block [202,203,214,215]. Mancarella et al. demonstrated that acidification of the cellular environment in response to hypoxia leads to uncoupling of ORAI1 from STIM1, effectively decreasing SOCE. This sensitivity to acidosis may be an important feedback mechanism to prevent toxic build-up of Ca^{2+} in response to hypoxia [202,203]. In essence, these data suggest that hypoxia induces SOCE during early stages of oxygen deprivation, while acidosis, which is a long-term consequence of hypoxia, may shut-down SOCE mediated Ca^{2+} entry to prevent intracellular Ca^{2+} accumulation and associated cell death (Fig. 7**B**). These data also highlight the potential differences between chronic and acute hypoxia, which may influence cellular ROS status and signaling pathways, including differences between acute HIF-1α and chronic HIF-2α activation [216]. What role this plays in SOCE inactivation in the context of an acidic tumor microenvironment, which has been generally described as a consequence of increased glycolytic flux of tumor cells, remains to be elucidated.

There is also an interesting link between hypoxic regulation of SOCE and manipulation of nutrient stress response pathways. The redox regulation of STIM1 has been associated with activation of AMPK in conditions of hypoxia (Fig. 7**B**) [200,201]. As mentioned above, increases in mitochondrial ROS in response to hypoxia are necessary to initiate SOCE [200,201]. The resultant Ca^{2+} response was shown to induce AMPK-phosphorylation by Calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) [201,217]. Although, it should be noted that oxidative stress has also been demonstrated to increase AMP levels to activate AMPK [218], the above studies demonstrate that Ca^{2+} activation of AMPK is an important alternate regulatory pathway, independent of the AMPK regulator liver kinase B1 (LKB1). While AMPK activation is commonly associated with stress adaptations, hyperactivation of this pathway via SOCE may also have deleterious consequences on normal cells. For example, in alveolar epithelial cells it was demonstrated that this hypoxia-mediated SOCE regulation of AMPK is deleterious and eventually leads to Na^+/K^+-ATPase downregulation *via* endocytosis. This results in the inhibition of alveolar fluid reabsorption and endothelial cell dysfunction [200]. However it appears that cells have the ability to by-pass this damaging pathway. Observations in dendritic cells isolated from $AMPK^{-/-}$ mice suggest that AMPK may also act in a negative feedback loop to blunt Ca^{2+} influx into cells. For example, loss of AMPK resulted in enhanced SOCE, and in higher expression of ORAI1 and Na^+/Ca^{2+} exchangers [219]. How this hypoxia-ROS-SOCE axis influences AMPK in cancer cells is unclear. AMPK is an important sensor of ATP availability and regulator of metabolism, thereby promoting an increase in catabolism and a decrease in anabolic pathways [220]. AMPK appears to have both pro- and anti-tumorigenic roles, and its positive role in cancer has been associated with eliciting metabolic flexibility of tumor cells under stress conditions [221,222]. A recent study demonstrated that the ROS-Ca^{2+}-CaMKKβ-AMPK axis plays a role in anoikis resistance of tumor cells. It was previously shown that matrix detachment of cell leads to redox stress due in part to changes in glucose uptake and a decrease in the NAD(P)H/NAD(P)+ ratios [223] and that AMPK can aid tumor cell survival during nutrient stress by resorting NADPH levels through activation of fatty-acid oxidation [224]. Sundararaman et al., showed that SOCE activation in response to matrix detachment precedes redox signaling, leading to AMPK phosphorylation in a CaMKKβ-dependent, LKB1-independent manner. These data suggest that AMPK regulation by SOCE may play an important role in the ability of cancer cells to adapt to anchorage-independence by promoting anoikis resistance and aiding spheroid formation, which are important aspects to tumor metastasis [225]. Whether Ca^{2+} signals or ROS spikes are the initial signal that drives this AMPK activation may require further investigations, however these studies demonstrate an example of the Ca^{2+}-ROS signaling axis that may be involved in cellular survival and adaptation of tumor cell metabolism in response to stress, such as hypoxia, loss of matrix attachment and nutrient deprivation.

3.2.2. Redox regulation of ORAI1 and ORAI3

The ORAI family consists of 3 isoforms, of which ORAI1 was demonstrated to be inhibited following cellular exposure to H_2O_2, with and IC_{50} of 34 μM [6]. Reduced channel conductance in response to H_2O_2 was dependent on C195, which is close to the plasma membrane on the extracellular side. Because the inhibition of ORAI1 channel activity required pre-incubation with H_2O_2 the authors argued that oxidation of C195 maintains the channel pore in a closed configuration prior to thapsigargin-mediated store depletion and hence prevents activation in response to store depletion. The lesser-studied ORAI2 isoform had a similar inhibition profile to ORAI1 [6]. In subsequent work the authors proposed that pretreatment of cells with H_2O_2 leads to intramolecular interaction of C195 with S239, locking the channel in a closed conformation [226]. It should be noted that the high concentrations of H_2O_2 (1 mM) used in this work may contribute to the irreversible state of this redox modification [226]. C143 and C126 have also been implicated with electrophilic interactions and inhibition of CRAC currents by curcumin and caffeic acid phenethyl ester [227].

Interestingly, C195 is absent in the ORAI3 isoform, and this difference may play a role in determining redox regulation of ORAI1/ORAI3 heteromultimers in a number of pathophysiological contexts, including cancer. This was first investigated in the context of T Helper (T_H) cells, where enhanced expression of ORAI3 were proposed to mediate redox insensitivity of the CRAC channel as T_H cells develop from naïve cells to effector cells [6]. Naïve cells, using ORAI1 as their major CRAC channel, were more sensitive to H_2O_2 mediated cell death and CRAC inhibition by H_2O_2. In contrast, as cells matured into effector T_H cells, they expressed a higher proportion of ORAI3 subunits. Presumably this leads to an increase in the ORAI3/ORAI1 ratio and a decrease in available C195 residues, resulting in channels that are less sensitive to redox-inhibition (Fig. 8). Increased ORAI3 expression in effector T_H cells correlated with a decrease in redox inhibition of channel conductance and an increase in proliferation and cytokine production [6]. Infection illustrates an important interplay between SOCE activation and ROS regulation, where pathogen associated peptides initiate IP_3-mediated Ca^{2+} store depletion and SOCE activation. The ensuing Ca^{2+} signals activate Nox2 at the cell surface *via* PKC to produce $O_2^{\bullet -}$ and H_2O_2 as part of the oxidative burst for the killing of the pathogen. Interestingly, a switch to a redox insensitive ORAI3/ORAI1 heteromultimer was also shown to occur in monocytes following bacterial infection [228]. A higher ORAI3/ORAI1 ratio was shown to reduce the Ca^{2+} amplitude [229], but resulted in a prolonged Ca^{2+} signal that was not inhibited by increases in ROS [228]. The authors proposed this to be a mechanism that ensures killing of the pathogen, but avoids extensive tissue damage in response to sustained ROS bursts.

Interestingly, the ORAI1/ORAI3 ratio also appears to influence the redox sensitivity of ORAI channels in cancer cells. It was shown that prostate cancer cell lines have a higher ratio of ORAI1/ORAI3 compared to primary human prostate epithelial cells [198]. The authors went on to show that an increased ORAI1/ORAI3 ratio makes prostate cancer cells more redox sensitive to SOCE inactivation by H_2O_2 [198]. H_2O_2 exposure of prostate cancer cells and normal prostate epithelial cells led to an initial increase in cytosolic Ca^{2+}, likely as a consequence of ROS-dependent TRP channel

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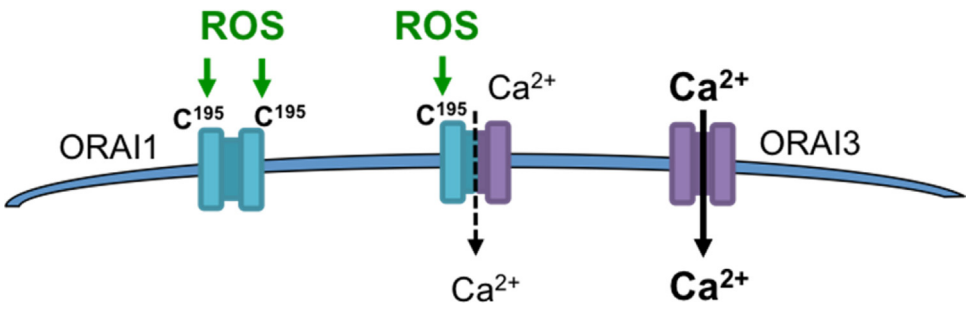

Fig. 8. Differential redox regulation between ORAI1 and ORAI3 due to the lack of C195 in ORAI3. Oxidation of ORAI1 leads to channel inhibition. The significance of ORAI1 redox inactivation, the ratio of ORAI1/ORAI3 expression, and the consequences of different ORAI heteromultimer conformations in cancer require further investigation.

activation, which differed between cancer and normal cells. This was followed by H_2O_2-dependent SOCE inactivation, as tested by store depletion using the SERCA pump blocker thapsigargin. The prostate cancer cell line LNCaP displaying a 10 fold lower H_2O_2 IC_{50} value compared to the normal prostate epithelial cell line hPECs. Although the prostate cancer cell DU145 displayed a similar IC_{50} to hPECs, the authors correlated a change in the ORAI1/ORAI3 expression ratio to increased susceptibility of cells to redox stress [198]. As mentioned previously, tumor cells are often more susceptible to ROS toxicity, This phenotype may be due to some tumor cells having higher intracellular steady state levels of ROS, which in turn places cells closer to the cytotoxic threshold of ROS, an observation that has triggered research into utilizing ROS-agents for chemotherapeutic applications [79–82,99]. Holzmann et al. proposed that ROS-mediated inactivation of ORAI1 may also contribute to higher sensitivity of prostate cancer cells to ROS, due to dampening of SOCE dependent pro-proliferative Ca^{2+} signaling [198,230]. The authors proposed that this altered ORAI1/ORAI3 expression ratio could provide a therapeutic opportunity to target the pro-proliferative actions of SOCE by carefully tuning redox-mediated inactivation to only affect tumor cells [198]. However, it should be pointed out that in all ORAI1/ORAI3 studies described above, it remains uncertain whether ORAI1 and ORAI3 form different quantities of two independent homohexameric channels or form heterohexameric ORAI1/ORAI3 channels with variable stoichiometries. Earlier studies provided evidence for native ORAI1/ORAI3 hetero-multimerization where these channels are not activated by store depletion, but instead by receptor-mediated production of arachidonic acid or its metabolite, LeukotrieneC_4 (LTC_4) [231–235]. In this context, the work by Holtzmann et al. [198] sharply contrasts with another study of prostate cancer, demonstrating increased levels of ORAI3 expression, in prostate tumor cells, which resulted in ORAI1/ORAI3 heteromultimeric channels that elicited Ca^{2+} entry in response to receptor stimulation in a store-independent fashion [236]. This was presumed to occur through production of arachidonic acid and/or LTC_4. While this study did not investigate the redox regulation of ORAI1/ORAI3 heteromultimeric channels, the authors proposed that these channels elicit Ca^{2+} signaling to drive proliferation, while a smaller proportion of ORAI1 homomeric channels mediate the classical SOCE pathway, which is pro-apoptotic [236]. Hence, an increase in ORAI3 expression would shift tumor cells towards pro-proliferative Ca^{2+} signaling. Differences in ORAI subunits expression have also been demonstrated for other tumor types. ORAI3 plays a significant role in mediating SOCE in estrogen receptor positive breast cancer cells, while estrogen receptor negative cells have CRAC channels that are largely composed of ORAI1 [182–184,189,237]. Reports of heterogeneity in ORAI1 expression within the same tumor also highlight the potential transient nature of SOCE regulation during tumor progression [213]. How relative ORAI1 and ORAI3 expression levels relate to the redox regulation of SOCE between histological subtypes and different areas of the tumor remains to be elucidated. However, it is reasonable to suspect that this could vary not only in terms of ORAI isoform expression, but also in terms of their exposure to exogenously and endogenously produced ROS, for example in response to hypoxia or enhanced immune cell infiltration.

ORAI1 may also be regulated by ROS in an indirect fashion. Feng et al. previously proposed that breast cancer cells express high levels of the Secretory Pathway Ca^{2+}-ATPase, SPCA2, which constitutively activates ORAI1 channels in a STIM and SOCE independent manner [181]. A recent study demonstrated that SPCA2 expression is enhanced in HCT116 colon cancer cells in response to hypoxia, on 3D spheroid growth, after exposure of cells to H_2O_2, and with agents that induce mitochondrial ROS (Antimycin A) and reactive nitrogen species (DETA NONOate) [238]. Although the investigators did not assess the activation of ORAI1 in this context, SPCA2 regulation by ROS/RNS, may be an indirect mechanism for the redox regulation of ORAI1 in cancer. Moreover, since mitochondrial dysfunction, aberrant mitochondrial redox signaling and ER stress are common occurrences in cancer, it is not unreasonable to suggest that these feed into the redox regulation of SOCE. As described further below, oxidation of the IP_3 Receptors has been demonstrated to influence their activity and hence alters the levels of Ca^{2+} in ER stores. For example, activation of IP_3R by H_2O_2 and consequential ER Ca^{2+} store depletion was shown to partially contribute to the activation of CRAC channels, indicating that SOCE is influenced at multiple levels by redox regulation [239]. How the oxidation of Ca^{2+} regulators at internal stores influence SOCE is only starting to be unraveled.

3.3. *Plasma membrane Ca^{2+} efflux pumps*

3.3.1. *Redox regulation of plasma membrane Ca^{2+} ATPase (PMCA)*

Contrary to the redox-activation of a number of Ca^{2+} influx channels, oxidation of the plasma membrane Ca^{2+} ATPase (PMCA), which extrudes Ca^{2+} from the cell, is largely inhibitory. This suggests that a global increase in oxidative state could cause enhancement of cytosolic Ca^{2+} levels. PMCA redox inactivation is thought to arise primarily as a consequence of oxidative stress in pathological conditions, such as neurodegeneration and reperfusion injury, leading to a buildup of cytosolic Ca^{2+}, degradation of the PMCA protein, and cell death [240,241]. This redox-dependent inhibition of PMCA may also be an avenue that mediates apoptosis in response to cytotoxic agents. In MCF7 breast cancer cells, redox-mediated apoptosis in response to the platinum analog ([Pt(O,O′-acac)(γ-acac)(DMS)]) was primarily initiated by the inhibition of PMCA and the subsequent increase in intracellular Ca^{2+} [242]. Activity of PMCA is also indirectly affected by oxidation of Calmodulin (CaM). Oxidation of CaM C-terminal methionines inhibits activation of PMCA, while still maintaining the PMCA-CaM interaction [243,244]. These data suggest that PMCA inactivation is a common consequence of redox stress and is important during

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apoptosis. It is unknown if tumor cells are more resistant to this inactivation. Studies have shown variable expression patterns of PMCA in cancer cells, which appear to be isoform-, cancer type- and stage-specific [245–250]. Enhanced expression of PMCA in tumor cells may be one mechanism by which tumor cells evade high Ca^{2+} buildup during apoptosis, and inhibiting this protein may be a way to initiate tumor cell death [248,250,251].

3.3.2. Redox regulation of Na^+/Ca^{2+} exchanger (NCX)

Contrary to PMCA, the Na^+/Ca^{2+} exchanger (NCX), which mostly elicits Ca^{2+} extrusion from the cell but can also work in the reverse mode to cause Ca^{2+} entry, is activated in response to thiol oxidation by H_2O_2. NCX activation together with Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA; discussed below) redox inactivation is thought to play a role in sarcoplasmic Ca^{2+} depletion and a decrease in contractile function of myocytes during heart failure [252]. Further studies are needed to determine if sub-lethal and localized changes in ROS can influence transient alterations in PMCA and NCX activity and if this plays a role in altering Ca^{2+} homeostasis and Ca^{2+}-mediated signaling in cancer cells.

4. Redox regulation of ER and mitochondrial Ca^{2+} modulators

4.1. The ER – mitochondrial interface

Controlled Ca^{2+} homeostasis of mitochondria is imperative for proper mitochondrial function. A number of proteins involved in the TCA cycle and electron transport chain rely on Ca^{2+} for their activity and can hence determine cellular metabolic flux and redox signaling [253]. However, as discussed below, high mitochondrial Ca^{2+} import together with increases in ROS are also essential for the onset of apoptotic cell death [254,255]. This influx of Ca^{2+} into the mitochondrial matrix is derived primarily from closely apposed ER, although Ca^{2+} entry channels at the plasma membrane also couple to mitochondrial Ca^{2+} uptake [256–258]. The importance of mitochondrial-ER cross talk has been emphasized by the discovery of Mitochondria-Associated Membranes (MAMs) as highly regulated hubs of ER-Mitochondria organellar interface. These areas of close proximity between the mitochondrial and ER membranes contain tethering proteins and channels that help facilitate ion exchange between the two organelles, most notably Ca^{2+}. MAMs are characterized by enrichment of ER-localized IP_3R/RyR receptors and SERCA pumps, and mitochondrial Voltage-dependent anion channel (VDAC) and the mitochondrial Ca^{2+} uniporter (MCU) in the outer and inner mitochondrial membrane, respectively (Fig. 4). These channels facilitate Ca^{2+} transfer between the ER and mitochondria and are known redox-activated proteins [52,89]. While the ER and mitochondrial membranes do not directly fuse, they are closely associated within 10–25 nm, and are tethered by proteins, such as Mitofusin 2 (MFN2), one of two mitochondrial fusion proteins [259,260]. Ca^{2+} levels within mitochondria are further regulated by Ca^{2+} efflux carried out by the mitochondrial Na^+/Ca^{2+} Exchanger (termed NCLX for its ability to exchange both Na^+ and Li^+ for Ca^{2+}) [261] and the mitochondria Permeability Transition Pore (mPTP) [262]. Mitochondrial Ca^{2+} homeostasis is fine-tuned by the activity of NCLX which extrudes Ca^{2+} from mitochondria into the cytosol, and in turn aids in maintaining mitochondrial redox homeostasis [263].

MAMs are increasingly being recognized as domains that are enriched in proteins contributing to both ROS and Ca^{2+} shuttling between the ER and mitochondria [9,264]. Interestingly, MAMs are high in oxidoreductase Ero1 levels, which is involved in disulfide bond formation during protein folding and an important regulator of the redox state of the ER [55]. Ero1 was shown to modulate both IP_3R-dependent Ca^{2+} release and the activity of MCU, therefore affecting Ca^{2+} shuttling from the ER to the mitochondria [265,266]. This regulation is tightly controlled by redox activation and the relative expression of Ero1. In scenarios of ER stress, the interaction between Ero1 and IP_3R and subsequent activation of Ca^{2+} release is an important mechanism in the initiation of apoptosis [267]. Similarly, Protein kinase RNA-like endoplasmic reticulum kinase (PERK) is localized to MAMs and shown to be an important mediator of ER-mitochondrial Ca^{2+} transfer in response to ROS-mediated ER stress [268]. Altered ER stress response pathways have been reported in cancer [269,270], but these have not been specifically linked with redox-sensitive Ca^{2+} regulatory pathways at the MAMs. However, one could speculate that this may be an important aspect to cancer cell adaptations in response to ER stress and the unfolded protein response.

4.1.1. Ca^{2+}-ROS interplay during apoptosis

The consequence of the Ca^{2+}-ROS interplay at the ER-mitochondrial interface is dependent on the amplitude and frequency of these signals. Most commonly, studies have focused on the large fluxes of Ca^{2+} and surges in ROS associated with apoptosis, which results in the opening of the mitochondrial Permeability Transition Pore (mPTP), mitochondrial membrane potential collapse, H_2O influx, mitochondrial swelling, and cytochrome c release (Fig. 9**A**) [271,272]. SERCA, IP_3R and VDAC are important mediators of Ca^{2+} transfer from the ER to the mitochondria during apoptosis [52,273,274]. Interestingly, a number of tumor suppressors have also been shown to associate with MAMs and been demonstrated to directly influence this Ca^{2+} shuttling [275–277]. While it has been shown that Ca^{2+} can directly initiate mitochondrial membrane permeabilization, through calcineurin-dependent dephosphorylation of BAD and its association with Bcl-xL [278], the coordinated, yet complex interaction of both Ca^{2+} and ROS, appears to be necessary for mPTP opening and apoptotic (and necrotic) cell death activation [279]. Madesh and Hajnoczky showed that $O_2^{\bullet-}$ promotes mPTP opening in a Ca^{2+}-dependent manner. VDAC is an important mediator of Ca^{2+} influx into the mitochondria in this context, as inhibition of VDAC inhibited $O_2^{\bullet-}$-dependent cytochorome c release [52]. While the exact mechanisms of redox regulation of mPTP are still being elucidated, adenine nucleotide translocase and cyclophilin D have been shown to have redox active cysteines, which control the opening of mPTP [280–282]. Cyclophilin D sensitizes the mPTP to Ca^{2+}, due to its direct interaction with F_0F_1-ATP synthase, dimers of which are thought to constitute the mPTP channel [283].

A number of mechanisms for the Ca^{2+} induced $O_2^{\bullet-}$ production in mitochondria have been proposed, including Ca^{2+} mediated increase in the activity of components of the TCA cycle and ETC, thereby accelerating electron leakage and $O_2^{\bullet-}$ production, and the role of Ca^{2+} on mPTP opening which may lead to further ROS production due to transient mitochondrial membrane potential depolarization and resultant increases in ETC $O_2^{\bullet-}$ production (for reviews see [5,271,282]). Moreover, ROS release from mitochondria appears to perpetuate mitochondrial ROS surges during apoptosis. This may be necessary for transmittal to adjacent mitochondria, eventually resulting in global activation of apoptotic cell death [284]. In contrast to the above, a decrease in mitochondrial Ca^{2+} may also interfere with normal function of the ETC to increase mitochondrial ROS production. An example is a recent study demonstrating that Ca^{2+} overload in mitochondria leads to Complex II disintegration from the ETC. While complex II is uncoupled from the ETC it remains active and produces ROS [53]. The investigators show that cardiolipin can interact with and sequester Complex II into protein aggregates, and that this is dependent on Ca^{2+}.

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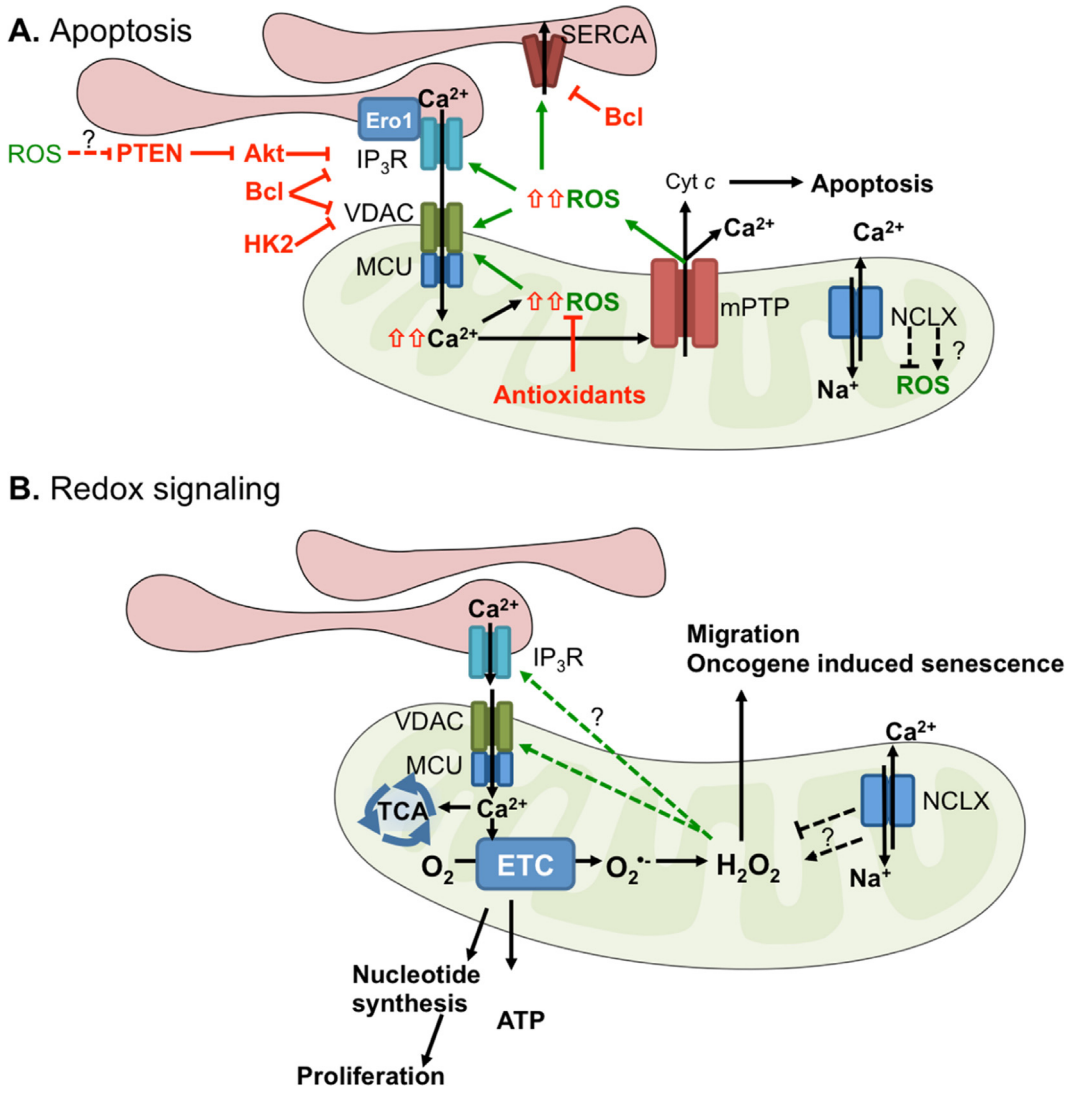

Fig. 9. Cancer cells have developed several mechanism to evade the pro-apoptotic ROS-Ca^{2+} cross talk at the ER-mitochondrial interface (**A**), while maintaining regulated mitochondrial Ca^{2+} influx that can regulate mitochondrial redox signaling to support tumor survival, growth and metastasis (**B**). **A.** Tumor cells have been shown to express anti-apoptotic proteins such as Bcl-2, Bcl-XL and HK2 to bind and inhibit IP3R and VCDA, and suppressing the Ca^{2+} transfer from the ER to the mitochondria, inhibiting Ca^{2+}-ROS dependent apoptosis [75–78,300–303]. **B.** The transfer of Ca^{2+} to the mitochondria is also important in maintaining ETC and TCA function and the ability of tumor cells to synthesize adequate levels of nucleotides for cell growth [299]. There are preliminary reports that this increase in Ca^{2+} ER- mitochondrial shuttling may be important for mitochondrial ROS production and redox mediated signaling to drive migration [304] and oncogene induced senescence [305]. Whether or not this increase in mitochondrial H_2O_2 exerts a positive feedback on IP_3R [85], VDAC and MCU in tumor cells remains to be determined. Studies have shown that activation of NCLX may increase mitochondrial ROS production, by decreasing Ca^{2+} levels in mitochondria [263,306]. However, NCLX knock down can also result in ROS generation, presumably due to its important role in fine tuning Na^+/Ca^{2+} balance [180]. The consequences of NCLX –mediated mitochondrial ROS production on cellular signaling are still unclear, but could have influence redox regulation of the CRAC channel ORAI [180].

Tumor cells adapt to evade apoptosis, with several reports demonstrating direct manipulation of mPTP opening in cancer cells [271]. These include modulation of cellular signaling to regulate mPTP opening, such as the dampening of PTEN mediated regulation of Ca^{2+} influx via IP_3R, as described below (reviewed in [271,279]). Tumor cells exhibit changes in Bcl apoptotic protein expression, and it is known that these can also alter the function of a number of Ca^{2+} channels including IP_3R (Fig. 9**A**) [285]. An example, is the interaction of the anti-apoptotic protein Bcl-2 with IP_3R. Binding of Bcl-2 to IP_3R slows IP_3-mediated release of Ca^{2+} from the ER, which results in a lowering of mitochondrial Ca^{2+} pools in cancer cells. This is sufficient to inhibit the initiation of apoptosis [75–78]. Increasing Ca^{2+} ER-mitochondrial shuttling may therefore be a therapeutic approach to enhance apoptosis initiation in cancer cells, and recent work has explored the use of a Bcl-2/IP_3R disrupter (BIRD-2) peptide to induce apoptosis in a number of hematological cancers and lung cancer studies [286–288]. In addition to the effects of dampening Ca^{2+} flux into mitochondria, cancer cells likely evade apoptosis by enhanced mitochondrial ROS scavenging, which could further inhibit mPTP opening.

4.1.2. *Ca^{2+}-ROS signaling at the ER – mitochondrial interface*

In comparison to ROS/Ca^{2+} surges during apoptosis, tightly controlled ROS and Ca^{2+} oscillations appear to be important in regulating cell signaling and the maintenance of energy production by mitochondria [289]. As such, small increases in H_2O_2 can initiate Ca^{2+} release from ER stores. For example, it was shown that H_2O_2-dependent oxidation and activation of PLCγ1 mediates IP_3R-dependent Ca^{2+} oscillations [290]. The investigators further demonstrated that EGF treatment of rat cortical astrocytes stimulated H_2O_2 production to initiate these oscillations. This may have

implications for growth factor signaling and ROS-Ca^{2+} crosstalk in cancer, where EGF is a major pro-proliferative pathway.

In general, it is accepted that physiologically relevant mitochondrial Ca^{2+} uptake occurs through shuttling of Ca^{2+} from the ER to the mitochondria and that this process is of importance for the TCA cycle generation of NADH, increase mitochondrial respiration and ATP production (Fig. 9**B**) [291–293]. A number of metabolic enzymes, electron transport chain proteins, substrate transporters, as well as changes in mitochondrial membrane potential are regulated by mitochondrial Ca^{2+} [253,294,295]. For example, Pyruvate dehydrogenase (PDH) activity is tightly regulated by Ca^{2+}. PDH-Phosphatase is activated by Ca^{2+}, which in turn dephosphorylates PDH, leading to an increase in PDH enzyme activity [296,297]. Ca^{2+}-dependent regulation of mitochondrial metabolic pathways is clearly an important aspect of normal cell function, and inhibition of Ca^{2+} transfer by blocking IP_3R results in decreased ATP production and activation of AMPK and autophagy [298]. In a recent study it was suggested that this switch to autophagy is not sufficient to ensure survival of tumor cells under stress conditions. It was proposed that mitochondrial Ca^{2+} uptake is required for maintenance of cancer cell nucleotide synthesis [299]. Thus Ca^{2+} transfer from the ER to mitochondria is an important pro-survival signal that is required for the synthesis of cellular building blocks by cancer cells, even if they rely less on mitochondrial ATP production. As described below, increased mitochondrial Ca^{2+}, as well as low mitochondrial Ca^{2+} have both been implicated with an increase in mitochondrial ROS production. This may occur through different mechanisms, including Ca^{2+} driven increases in ETC flux, which enhances electron leakage and $O_2^{\bullet-}$ production. Alternatively, a decline in mitochondrial Ca^{2+} could lead to a decrease in TCA cycle generation of reducing equivalents, thereby inhibiting the ROS scavenging ability of mitochondria [253]. The observation that mitochondrial Ca^{2+} can also regulate ROS production may be one mechanism by which Ca^{2+} initiates a subsequent mitochondrial redox-signaling loop to further ensure tumor cell survival. As with plasma membrane channels, the levels and duration of Ca^{2+} mitochondrial influx determine the cellular consequences of the ER-mitochondrial ROS-Ca^{2+} cross talk (Fig. 9).

4.2. Redox regulation of inositol 1,4,5-trisphosphate (IP_3) receptors

As mentioned above, maintenance of physiologically relevant Ca^{2+} levels in the mitochondria is important for proper mitochondrial function. The IP_3R and the related RyR are the primary regulators of ER/SR Ca^{2+} release into the cytoplasm and Ca^{2+} transfer from the ER (or SR) to the mitochondria. The latter is important in facilitating large surges of Ca^{2+} transfer during apoptosis, as well as more transient Ca^{2+} oscillations to maintain ATP production. Like RyR, IP_3R is a demonstrated target for redox-mediated regulation [307,308]. Different redox modifications have also been observed for IP_3R. Glutathionylation induced by Diamide and H_2O_2 treatment enhances IP_3R Ca^{2+} release in response to Ca^{2+} [309]. In addition, disulfide bond formation of ER luminal cysteines (C2496, C2504, or C2527) was demonstrated to lead to IP_3R mediated Ca^{2+} release, due to the dissociation of IP_3R1 from the thioredoxin ERp44, which inhibits IP_3R under reducing conditions [310], Moreover, direct action of $O_2^{\bullet-}$ on IP_3R activity was demonstrated using a xanthine–xanthine oxidase delivery system, that presumably resulted in the oxidation of cytosolic residues of the receptor [311]. The authors argued that any mitochondria-derived $O_2^{\bullet-}$ may not have the ability to reach the IP_3R and instead needs to be first dismuted to H_2O_2. Indeed a follow-up study demonstrated that IP_3R Ca^{2+} oscillations yield localized increases in H_2O_2 at MAMs and that this results in a positive redox feedback on IP_3R activation [85]. Interestingly, these localized H_2O_2 "nano-domains" are thought to originate as a consequence of Ca^{2+} induced H_2O and K^+ influx into the matrix, which results in mitochondrial cristae deformation and potential rearrangement of ETC components into supercomplexes that could be involved in the observed localized H_2O_2 increases [85,312]. It would be of great interest to determine if such Ca^{2+}-dependent mitochondrial cristae shape changes are a phenotype of cancer cells that regulate redox and Ca^{2+} signaling, or if these changes are altered to prevent Ca^{2+} influx into mitochondria during the process of apoptosis. Moreover, this may have implications for ETC function and metabolism of cancer cells. As such, it has been shown that IP_3R-dependent Ca^{2+} transfer from the ER to the mitochondria sustains ATP production while maintenance of ATP levels ensures that AMPK-dependent autophagy is suppressed in nutrient-rich environment [298]. IP_3R-mediated Ca^{2+} transfer to mitochondria is also linked to the Ca^{2+}-dependent activation of PDH phosphatase, which in turn activates PDH, leading to increased Acetyl CoA flow into the TCA cycle and respiration [298]. Conversely, when this Ca^{2+} flow from the ER to the mitochondria is suppressed, ATP levels fall and autophagy is initiated as a survival mechanism. It should be pointed out that there are conflicting reports on the role of IP_3R on both promoting and suppressing autophagy, which is likely dependent on nutrient availability (i.e. culture conditions), cell type and feedback mechanisms of Ca^{2+} signaling that control autophagy regulators such as Beclin I [164]. Many tumor cells are thought to survive without oxidative phosphorylation, relying primarily on aerobic glycolysis. As mentioned above, a recent study found that tumor cells are exquisitely sensitive to a block in ER to mitochondrial Ca^{2+} transfer. While this resulted in phosphorylation of AMPK and initiation of autophagy in both normal and cancer cells, lack of Ca^{2+} release through IP_3R or inhibition of mitochondrial Ca^{2+} uptake by blocking MCU led to increased cell death specifically in tumor cells. This was shown to be due to initiation of necrosis, and the authors concluded that inhibition of ER-mitochondrial Ca^{2+} transfer leads to decreased mitochondrial function and a lack of nucleotide production, important building blocks for proliferating cancer cells [299]. With the recognition that metabolic pathways besides aerobic glycolysis are important for the maintenance of tumor cell macromolecules, the importance of altered mitochondrial function in tumors is of great interest. The role for the IP_3R in maintaining adequate Ca^{2+} balance within mitochondria of cancer cells is starting to emerge. The interplay between ROS and IP_3R activity will no doubt be an important regulatory mechanism in this context.

In addition to direct oxidation of IP_3R, ROS can influence IP_3R activity indirectly. This was demonstrated in breast cancer cells, where cells with mitochondrial dysfunction and increased ROS production displayed enhanced expression of the chemokine CXCL14, as a consequence of ROS and AP1-dependent regulation of the *CXCL14* promoter. In turn, CXCL14 induced IP_3R-mediated Ca^{2+} release, which was necessary for ROS-dependent breast cancer cell migration [313]. IP_3R was shown to interact and be regulated by a number of kinases and phosphatases, including the Akt and PTEN signaling axis, where IP_3R phosphorylation by Akt has been shown to decrease IP_3R3 activity and suppress pro-apoptotic Ca^{2+} release [275,276,314]. Conversely, during induction of apoptosis association of IP_3R with the phosphatase PTEN is enhanced at MAMs and leads to de-phosphorylation of IP_3R, resulting in increased mitochondrial Ca^{2+} [276]. PTEN is a known tumor suppressor commonly lost in a number of cancer types and re-expression of PTEN has been shown to restore IP_3R mediated apoptosis [315]. Interestingly, PTEN is a direct target for redox mediated signaling, as cysteine oxidation leads to decreased PTEN activity, and redox mediated inactivation has been observed in cancer cells with higher redox status [98,316,317]. Whether PTEN oxidation and a concomitant decrease in IP_3R mediated Ca^{2+} release into mitochondria are

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involved in apoptosis resistance of tumor cells, remains to be elucidated.

4.3. Redox regulation of voltage-dependent anion channel (VDAC)

VDACs are localized to the outer mitochondrial membrane, are commonly localized in MAMs, facilitate shuttling of metabolites and ions across the outer mitochondrial membrane and may serve as a channel that allows $O_2^{\bullet-}$ release from the mitochondria [5,318]. Although VDACs are implicated in mPTP opening, studies from knockout cells suggest that VDACs do not constitute the pore forming unit of the mPTP. Cysteine residues of VDAC face the intermembrane space, are located in the relatively accessible pore region and are potentially oxidized by $O_2^{\bullet-}$ generated from electron leakage from the ETC at complex III [318,319]. Screens for oxidized thiols have identified VDACs as potential redox modified proteins, however the consequences of specific redox modifications of different VDAC isoforms are only starting to be interrogated and it is unclear if these play a role in VDAC activity or participate in altered VDAC function in a pathophysiological situation like cancer. As described briefly below, the role of VDAC may be primarily by facilitating mitochondrial Ca^{2+} uptake to enable mitochondrial ROS production, which is particularly evident during the process of apoptosis and necrosis. For example, pharmacological and antibody blockade of VDAC inhibited $O_2^{\bullet-}$-dependent cytochorome c release [52]. Of the three VDAC isoforms, VDAC1 is thought to be the primary channel involved in Ca^{2+} loading of mitochondria during apoptosis [274]. Similar to IP_3R, VDAC1 activity is inhibited by binding to anti-apoptotic proteins like hexokinase I and Bcl-2 family members, with Bcl-XL being more important to VDAC1 inhibition than Bcl-2 [300–302]. To increase apoptotic cell death in cancer cells, studies have focused on inhibiting the interaction between anti-apoptotic proteins and VDAC using specific peptides to prevent protein–protein interactions. In addition, it has been shown that forced VDAC expression and pharmacological approaches that enhance VDAC activity can enhance apoptosis of cancer cells [320–322]. Interaction of VDAC with Hexokinase (HK) may decrease apoptosis. This may be particularly important in cancers demonstrating high glycolytic flux and overexpression of HK2. Dissociation of HK2 lifts its inhibitory action on the channel, leads to VDAC oligomerization and allows for initiation of apoptosis [303]. Again, disrupting this interaction has been proposed as a therapeutic strategy. However, in addition to prevention of VDAC-mediated apoptosis, the interactions of VDAC with anti-apoptotic proteins may also serve to fine tune VDAC-mediated Ca^{2+} mitochondrial uptake thus regulating mitochondrial ROS signaling. For example, in non-small cell lung carcinoma the antiapoptotic protein Mcl-1, was shown to bind VDAC1 and 3 isoforms and this interaction was necessary for Ca^{2+}-driven ROS production that was important for lung cancer cell migration, but not proliferation [304]. Therein, VDAC facilitates non-canonical signaling of anti-apoptotic proteins and connects Ca^{2+} to mitochondrial redox signaling to regulate pro-metastatic phenotypes.

4.4. Redox regulation of mitochondrial Ca^{2+} uniporter (MCU)

The MCU is a Ca^{2+}-selective channel localized in the inner mitochondrial membrane and mediates Ca^{2+} uptake into the mitochondrial matrix, which is dependent on mitochondrial membrane potential (ΔYm) and pH [323,324]. MCU comprises the pore-forming unit of a complex of proteins that includes regulatory proteins involved in gating of MCU (for review see [325]). The role of MCU-regulated mitochondrial Ca^{2+} uptake on maintaining mitochondrial bioenergetics is dependent on the relative metabolic activity of specific tissues, as demonstrated in MCU knockout animals, where loss of MCU has little effect on Oxidative Phosphorylation of mouse embryonic fibroblasts. On the contrary, pronounced effect on mitochondrial bioenergetics are observed following MCU loss in skeletal and cardiac tissues under stress condition [326,327]. MCU channel activity is regulated by a number of accessory proteins, including Mitochondrial Ca^{2+} Uptake 1 (MICU1), a Ca^{2+} regulated gate keeper that modulates mitochondrial Ca^{2+} uptake by Ca^{2+} dependent inhibition of MCU [324,328]. Loss of MICU1, results in sustained Ca^{2+} influx into mitochondria leading to increased mitochondrial ROS production [328]. Depending on the levels of Ca^{2+} influx and ROS production this can either lead to redox-mediated signaling, inhibition of Oxidative Phosphorylation, or increased susceptibility to apoptosis [328]. It appears that the MCU regulatory proteins may also determine MCU activation in a cell type specific manner [329]. Loss of the positive MCU Regulator 1 (MCUR1), disrupts mitochondrial function in vascular endothelial cells and leads to induction of autophagy, as an apparent compensatory mechanism [329]. The function of MCU and its regulators in cancer is only starting to be unraveled. In one study, IP_3R2 and MCU were identified as two important mediators of oncogene-induced senescence and replicative senescence using an shRNA screen [305]. Knockdown of these proteins caused tumor cells to exit senescence, and caused a concomitant decrease in mitochondrial Ca^{2+} influx. It was proposed that the maintenance of mitochondrial Ca^{2+} and ROS by MCU and IP_3R2 are required for cells to stay senescent [305].

There is conflicting data on the changes in MCU expression between cancer types and the respective involvement of MCU in regulating mitochondrial function in this context. Decreased MCU expression in colon and prostate cancer was associated with high levels of the micro RNA miR-25. Low MCU levels correlated with reduced susceptibility to apoptosis, due to lack of MCU-mediated Ca^{2+} mitochondrial uptake [330]. Conversely, increased MCU and low MICU1 expression has been associated with poorer survival outcomes in breast cancer patients [331]. Interestingly, changing expression of either protein did not significantly alter clonogenic survival of MDA-MB-231 breast cancer cells in response to various stressors, such as nutrient deprivation or chemotherapy, while normal breast epithelial cells were dependent on the presence of MCU and MICU1 for cell survival [331]. Other investigators similarly demonstrated that high MCU expression correlates with increasing stage of breast cancer and an invasive phenotype, yet that decreasing MCU expression has little effect on inhibiting cell proliferation or viability [332,333]. It was shown that loss of MCU does not influence caspase-dependent apoptosis, but that ionomycin-induced caspase-independent cell death is increased in response to MCU loss in estrogen receptor negative and basal-like breast cancers [332]. This may be related to histological subtype as, triple negative cancers appear to require MCU for tumor growth, as described below [334].

Interestingly, recent studies suggest that MCU may control the interplay between Ca^{2+} and redox signaling emanating at the mitochondria. Reducing either MCU or MICU1 expression diminishes mitochondrial $O_2^{\bullet-}$ flashes in response to hyperosmotic stress [335]. This study highlights that changes in these signaling flashes are initiated and dependent on synergistic, physiologically relevant increases in mitochondrial Ca^{2+} and ROS. These flashes were shown to be important for activating the MAP Kinase pathway as both Jnk and Erk phosphorylation were inhibited with the use of a mitochondrial ROS scavenger [335]. MCU was also shown to be important for the generation of mitochondrial ROS flashes that drive oxidation and inactivation of Rho-1 and consequential actin mediated wound-closure in a *C.elegans* wound healing model [86]. The investigators showed rapid increase in intracellular Ca^{2+} following wounding, which is buffered by the mitochondria, specifically through the MCU. MCU was necessary to elicit the production of mitochondrial ROS sparks, and the investigators concluded that

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the primary reactive species eliciting redox signaling is $O_2^{\bullet-}$, as mutants lacking SOD isoform expression displayed enhanced wound healing. Due to its short-lived nature, this would imply that mitochondria are closely associated with Rho-1 at the actin cytoskeleton. It is unclear if MCU plays a similar role in regulating mitochondrial ROS flashes to drive proliferative and migratory cell signaling in cancer cells. A recent study investigating the role of MCU in triple negative breast cancer progression indicates that this may indeed be relevant in cancer. Knock-down of MCU expression decreased cell migration and invasion and inhibited tumor formation and metastatic spread of triple negative breast cancer cells in an *in vivo* xenograft model [334]. Moreover, MCU-mediated mitochondrial Ca^{2+} influx and concomitant ROS-increases were shown to be necessary for HIF-1α activation, indicating an important role of MCU in regulating mitochondrial redox signaling in cancer cells [334]. In addition, it was shown that MCU is necessary for breast cancer cell migration and that this is dependent on SOCE activation [333].

While it appears that MCU can initiate redox changes at the mitochondria, reciprocal regulation of MCU channel activation by ROS has not been investigated in great detail. Direct oxidation of MCU or its interacting partner has not been described. The redox sensitive CaMKII has been shown to activate MCU-mediated Ca^{2+} currents in response to ischemia reperfusion injury, leading to myocardial cell death [336]. This could represent one indirect mechanism of MCU redox regulation in response to oxidative stress.

4.5. Redox regulation of mitochondrial Na^+/Ca^{2+} exchanger (NCLX)

The molecular identity of the mitochondrial Na^+/Ca^{2+} Exchanger (NCLX) was recently identified and shown to be an important regulator of Ca^{2+} efflux from the mitochondria [261]. Since NCLX mediated Ca^{2+} efflux is dependent on cytosolic Na^+ levels, cytosolic Na^+ homeostasis can influence mitochondrial Ca^{2+} levels. Interestingly, under certain pathological conditions an increase in cytosolic Na^+ has been shown to mediate decreased mitochondrial Ca^{2+} levels that in turn yield increases in mitochondrial ROS production. This is in apparent contrast to the above mentioned studies that show synergistic increases between mitochondrial Ca^{2+} influx and ROS generation. In a heart disease model it was shown that reduced mitochondrial Ca^{2+} as a consequence of increased cytosolic Na^+ build-up, results in decreased mitochondrial bioenergetics and TCA cycle activation [306,337]. This effectively decreased NAD(P)H:NA(P)D^+ ratios and abrogated the reducing potential of the mitochondria, leading to a build-up of mitochondrial ROS [306]. These data implicate NCLX as a regulator of mitochondrial ROS generation in pathophysiological contexts. Indeed, it was demonstrated that forced expression of NCLX could increase mitochondrial oxidation of the redox sensitive RoGFP probe and abrogate histamine-induced increases in mitochondrial Ca^{2+} and NAD(P)H, presumably through its role in decreasing mitochondrial Ca^{2+} levels and hence inhibiting matrix dehydrogenase activity [263]. Recently, the consequences of this mitochondrial ROS-NCLX interplay were further explored using a model of NCLX knockdown [180]. In this study, it was demonstrated that knockdown of NCLX increases mitochondrial ROS production by enhancing mitochondrial Ca^{2+} levels and that this mediates inhibition of the CRAC channel ORAI1. Interestingly, ER Ca^{2+} store depletion resulted in both SOCE activation and cytosolic Na^+ accumulation, suggesting an important interplay between SOCE and NCLX regulation [180]. While knock-down of NCLX had no effect on ORAI1-STIM1 interaction, it blunted CRAC channel activity, which was demonstrated to be dependent on an increase in mitochondrial ROS generation. Scavenging of mitochondrial H_2O_2 with a mitochondrial targeted catalase construct could rescue SOCE. It was shown that in response to NCLX knockdown, increased mitochondrial H_2O_2 inactivates ORAI1 through oxidation of C195, as the ORAI1 C195S was insensitive to NCLX expression loss [180]. The above two studies demonstrate that regulation of Ca^{2+} transients by NCLX is important in modulating mitochondrial ROS production, and that both NCLX-dependent decreases or increases in mitochondrial Ca^{2+} can result in enhanced mitochondrial ROS production. This may have implications for pathological conditions where NCLX activity is altered. Whether or not NCLX expression or Na^+-mediated NCLX activity is altered in a majority of tumor cells remains to be investigated. However, this could have direct consequences on pro-tumorigenic redox signaling or through redox control of SOCE-regulated pathways. The potential role of NCLX in fine-tuning the cross talk between ER-mitochondria Ca^{2+} transfer and SOCE regulation were studied in a model of malignant melanoma cells [338]. Enhanced trans-mitochondrial Ca^{2+} flux was observed in metastatic cells compared to non-metastatic clones, and this was correlated with enhanced SOCE and activation of the pro-survival Akt pathway [338]. Pharmacological inhibition of NCLX blocked this trans-mitochondrial flux and SOCE activation. This study also highlighted previous work demonstrating that nano-domains of close interactions between mitochondria and the plasma membrane play a central role in the regulation of plasma membrane CRAC channels by supporting mitochondrial Ca^{2+} buffering at the plasma membrane thus relieving Ca^{2+}-dependent inhibition of CRAC channels [256–258].

4.6. Redox regulation of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)

The SERCA pump is an important regulator of ER Ca^{2+} homeostasis and involved in rapidly refilling stores in response to receptor-mediated store depletion. Like the IP_3Rs, SERCA pump family (SERCA1-3) members are important regulators of ER Ca^{2+} during apoptosis and integral in maintaining Ca^{2+} levels to support the protein synthesis and folding machinery of the ER, including calreticulin and calnexin [339]. Hence, ER Ca^{2+} imbalance is a feature of ER-stress and the unfolded protein response [340,341]. The expression of SERCA isoforms during tumorigenesis appears to be dependent on tumor stage and type. Several studies have demonstrated direct redox modification of this pump, and the consequence on SERCA activity appears to be dependent on the specific modification present as well as the level of oxidant stress. While these redox changes in cancers have not been explored in depth, there is precedent to suggest that redox mediated inactivation of SERCA could be a phenotype of cancer cells to avoid Ca^{2+} mediated cell death. Screening for SERCA oxidation-sensitive cysteines revealed that a number of thiol residues susceptible to redox modification in response to aging and peroxinitrite exposure, with C344 and C349 forming intra-molecular disulfide bonds [342]. Age related cysteine modifications were associated with a decrease in SERCA activity during this process.

Illustrating that different modifications have diverse actions on SERCA activity, S-glutahtionylation of C669 and C674 activated SERCA in the carotid artery in response to NO. This was shown to be mediated by the action of $ONOO^-$, and dependent on the presence of adequate levels of GSH [343]. This highlights that the GSH/GSSH pool within cells is an important determinant for protein redox modification. S-glutahtionlylation of SERCA was also recently shown to occur during the unfolded protein response, with loss of the ER glutathione *S*-transferase Pi expression resulting in cells being more sensitive to ER-stress causing agents [344]. SERCA Tyrosine nitration was also demonstrated in models of cardiovascular disease [345,346]. In this case nitration may be associated with decreases in SERCA activity. Disruption of these oxidative modifications has been proposed as a potential therapeutic strategy to alter

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SERCA activity in disease models [347]. This could have potential use to either induce activity for promotion of apoptosis in cancer cells, or inhibit SERCA mediated apoptosis in cases of cardiovascular disease.

Like IP_3R, SERCA1 and SERCA2b have also been shown to interact with a number of anti-apoptotic factors, including Bcl-2, which inhibit SERCA-mediated Ca^{2+} ER uptake by eliciting a conformational change in the SERCA proteins [348,349]. This may also result in protein degradation. Similarly, in cells expressing mutant K-Ras, decreased IP_3R3 and SERCA2b protein expression were observed [350], suggesting an overall dampening of the ER-mitochondrial Ca^{2+} transfer through oncogenic inhibition that may provide anti-apoptotic benefits to cancer cells. The tumor suppressor p53 was recently shown to initiate cell death in response to cytotoxic agent Adriamycin, ROS and ER stress, by directly interacting with SERCA at MAMs, and activating SERCA to increase ER Ca^{2+} uptake [351,352]. This non-canonical action of p53 outside the nucleus in turn led to mitochondrial Ca^{2+} accumulation and initiation of apoptosis. This p53-mediated activation was shown to correlate with a decrease in SERCA oxidation, although the mechanisms behind this loss of oxidation are unclear [352]. Given that a number of cancers display high frequency p53 mutations, this may be one mechanism by which apoptosis is avoided in tumors. Indeed the investigators demonstrated that common p53 mutants are not able to increase SERCA mediated mitochondrial Ca^{2+} accumulation and cell death.

5. Conclusion

The interplay between Ca^{2+} and oxidants in cancer is only starting to be unraveled. From the above work we can conclude that cancer cells successfully utilize this crosstalk to initiate pro-tumorigenic signaling pathways that may be influenced by oncogene expression, growth factor signaling and changes in the tumor microenvironment (e.g. hypoxia, nutrient deprivation). Moreover, tumor cells appear to blunt the large surges in ROS and Ca^{2+} associated with apoptosis. As described in several examples above, the spatio-temporal nature of the ROS-Ca^{2+} interplay is of importance to the eventual cellular response, and future studies will need to focus on these important aspects to gain a better picture of the complex nature of this interplay in cancer. While methods for fast, precise and accurate measurements of Ca^{2+} levels in different organelles are fast improving, similar strategies for spatio-temporal ROS measurements are lagging behind highlighting the need of developing high resolution imaging tools to track ROS changes in specific organelles and specific regions of the cell. In addition, studies will need to focus on the role of the tumor microenvironment in manipulating these changes in Ca^{2+} and ROS signaling. These will include the influence of tumor-associated cells including fibroblasts and macrophages, and further exploration of the role of chemical and physical cues of the tumor environment. Since many modulators of Ca^{2+} signaling are potential "druggable" targets, understanding their regulation by altered redox-signaling is imperative in the context of cancer. Utilizing the knowledge gained by understanding the relative alterations in redox-Ca^{2+} homeostasis in tumor cells compared to normal cells, will hopefully allow us to exploit these changes to promote pro-apoptotic pathways for the purpose of cancer therapy.

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